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(54) Title: MULTI-STEP SYNTHESIS OF TEMPLATED MOLECULES

(57) Abstract: Disclosed is a method for the manufacture of a library of complexes. The complexes comprise templated molecules attached to the template which directed the synthesis thereof. The templated molecules are produced in a step-by-step fashion which provides for a high local concentration of reactive groups involved in the formation of connections between the individual components of the template molecule.

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Multi-step synthesis of templated molecules.

Technical Field

5 The present invention relates to a method for the manufacture of a library of complexes. Each complex in the library comprises a templated molecule attached to the template which directed the synthesis thereof. The library of the invention is useful in the quest for new biological active compounds, such as drugs.

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Background art

The generation of molecules carrying new properties remains a challenging task. Recently, a number of procedures have been suggested that should allow a more efficient generation and screening of a larger number of molecules. The approaches taken involve the encoding and/or templating of molecules other than natural biopolymers such as peptide, RNA and DNA. These approaches allow the researcher to generate and screen a huge number of molecules in a short time. This should lead to better molecules carrying the desired properties.

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The central dogma of biology describes the one-way flow of information from DNA to RNA to protein. Recently, methods such as phage display, peptides-on-plasmids, ribosome display and mRNA-protein fusion have been developed, allowing the transfer of information from the level of protein/peptide to RNA or DNA. This has enabled the use of molecular evolution to be applied on huge numbers of peptides that are exposed to an enrichment process, where after the enriched pool of molecules (enriched for a particular feature, such as binding to receptor protein) are amplified, by exploiting information flow from the peptide to DNA and then amplifying the DNA.

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More recently, approaches have been developed that allow the encoding of polypeptides and other biochemical polymers. An example of this approach is

disclosed in US 5,723,598, which pertains to the identification of a biochemical polymer that participates in a preselected binding interaction with a target to form a binding reaction complex. The prior art method encompasses the generation of a library of bifunctional molecules. One part of the bifunctional molecule is the biochemical polymer and the other part is an identifier oligonucleotide comprising a sequence of nucleotides which encodes and identifies the biochemical polymer. Following the generation of the library of the bifunctional molecules, a partitioning with respect to affinity towards the target is conducted and the identifier oligonucleotide part of the bifunctional molecule is amplified by means of PCR. Eventually, the PCR amplicons are sequenced and decoded for identification of the biochemical polymer. This approach suffers from the draw-back that it is necessary with a laborious decoding step following each round of selection. Thus the flow of information from the identifier sequence to the biochemical polymer is restrained.

Halpin and Harbury have in WO 00/23458 suggested an improvement to the approach stipulated immediately above, wherein the molecules formed are not only identified but also directed by the nucleic acid tag. The approach is based on the traditional split-and-combine strategy for synthesis of combinatorial libraries comprising two or more synthetic steps. Plurality nucleic acid templates are used, each having at one end a chemical reactive site and dispersed throughout the strand a plurality of codon regions, each of said codon regions in turn specifying different codons. Separately, each of the strands, identified by a first codon region, is reacted at the chemical reaction sites with specific selected reagents. Subsequently, all the strands are pooled and subjected to a second partitioning based on a second codon region. The split-and-combine method is conducted an appropriate number of times to produce a library of typically between 10^3 and 10^6 different compounds. The split-and-combine method is cumbersome and generates only a relatively small library.

The present invention aims at obtaining a library of compounds which are not only encoded by a suitable tag attached to each compound, but also directed. The directed synthesis of the compounds of the library allows for renewed synthesis of templated molecules following a selection round. Furthermore, the present invention increases the probability of connection of molecular entities eventually appearing in the templated molecule due to a higher local concentration of the reactive groups involved in the formation of the connection.

10 Disclosure of the invention

The present invention relates to a method for the manufacture of a library of complexes comprising templated molecules, said method comprises the steps of

- 15 a) providing a plurality of different templates comprising a number of coding regions and a reactive group, wherein each coding region of a specific template specifies a unique codon,
- b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of each building block complements a unique codon of a template, and
20 the functional entity comprises at least one reactive group,
- c) contacting the plurality of different templates with a subset of the plurality of different building blocks, said subset having anti-codons which complement the unique codons of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codons to the unique codons of the templates,
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- d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,
- e) contacting under conditions allowing specific hybridisation, the plurality of different templates harbouring the nascent templated molecules
30 with a further subset of the plurality of building blocks, said subset having anti-codons complementary to the unique codons of a coding

region in the vicinity of the coding region harbouring the nascent templated molecules,

- f) allowing the functional entities of the subset of further building blocks to form a chemical connection to the nascent templated molecules,
- 5 g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,
- h) optionally repeating steps e) through g),
- 10 i) obtaining a templated molecule attached via the linker one or more building blocks to the template which directed the synthesis thereof.

The present invention allow for a multi-step templated synthesis of a library of molecules without the need for a laborious and time consuming split step of the traditional split and-mix-method. The method furthermore provides for the possibility of bringing the functional entities of building blocks in close proximity, thus allowing facilitated connection of functional entities in the vicinity of each other. The bringing in close proximity of building blocks provide for an increased local concentration of the active chemical groups, thus increasing the probability that two reactive groups will be so close that a reaction actually will occur.

The various templates of the present invention are in general constructed to follow a general scheme. According to the scheme, a number of coding regions are provided on the template. In turn, each of the coding regions specifies one or more unique codons. Thus, a specific template comprises a given number of unique codons. The plurality of templates can, taken as a whole, be characterized as a library comprising the total amount of the different combinations of unique codons possible, or any subset thereof. The coding regions are suitable positioned in a linear sequence, such that the individual coding regions are positioned immediately next to each other. In some embodiments, it may be of advantage to use a branched template to ensure

proximity of reactive groups, the introduction of catalysts in the vicinity of the reactive groups or the introduction of as third reactant.

5 Besides the coding regions, the templates used in the present invention include a reactive group. The reactive group comprised by the template may be covalently or non-covalently attached to the template. Covalent attachment may be preferred when the templated molecule is to be effectively attached to the template, because a covalent bonding will allow affinity selection using more harsh conditions. The covalent attachment of the reactive
10 group may be done at a terminal region of the template or at a central region thereof. In an aspect of the invention, the reactive group is non-covalently attached to the template using a complementing element hybridised to the template. More particularly, it is preferred that the reactive group of the template is part of a building block hybridised to the template.

15 The coding regions may be spaced with a suitable spacer region. The spacer region may be an identifier for the coding region or may be a region not carrying any information but serving to bring the functional entities into the desired proximity or to provide the template with a desired physical characteristic like a stiff connection of coding regions, or alternative, a flexible connection between two coding regions.
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The template may comprise flanking regions. One of the flanking regions can in an aspect of the invention serve to immobilize the template to a surface of
25 a solid support. In another aspect of the invention the flanking region can encompass a signal group, such a flourophor or a radio active group, to allow a direct detection of the presence of the template.

The plurality of templates used in the present invention may in one embodiment be represented by the general formula:
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$$F-(\text{Coding region } 1)-(S^1)-(\text{Coding region } 2)-(S^2)\dots-(\text{Coding region } n)-(S^n)-F'$$

Wherein

Each of Coding region 1 through Coding region n independently specifies
m unique codons,

- 5 F and F' are optional flanking regions,
S¹ to Sⁿ are optional spacing groups,
n is an integer of at least 2, and
m is an integer of at least 1.

- 10 The unique codons of the templates are preferably composed of a sequence of nucleic acid monomers, such as nucleotides. Each codon is unique in the sense that within the same coding region no other codons have an identical sequence and length of nucleic acid monomers. Preferably, a unique codon does not have a corresponding sequence anywhere in the plurality of templates. To avoid hybridisation between individual templates it is also desirable to design each of the unique codons such that the complementary sequence thereof does not exist on any other templates.

- 20 The number of coding regions may be selected in accordance with *inter alia* the number of the desired final templated compounds, the building blocks available and the envisaged structure of the templated compound. According to the invention the number of coding regions is preferably at least 3 to achieve the desired diversity. The upper limit for the number of coding regions has not yet been elucidated; however it is believed that a number exceeding 100 may give practical problems. Generally, it is preferred to use
25 templates having between 3 and 50 coding regions, more preferably between 3 and 30 and still more preferred between 4 and 15.

- 30 Within each of the coding regions the number of unique codons may be selected according to the need for diversity. The number of unique codons in each of the coding regions may be similar or different. The number of unique codons can be as low as one. This may be the choice when a so-called scaffold

fold is involved in the evolving templated molecule. The upper limit for the number of unique codons may be chosen quit high as long as specific hybridisation of oligonucleotides of the anti-codons to their complements on the templates occurs. An example of an upper limit may be 10,000, but may be
5 chosen below this limit or above according to the need.

The building blocks essential for the present invention, are generally composed of three elements, viz. an anti-codon, a functional entity, and a linker which connects the anti-codon and the functional entity. The anti-codon is a
10 sequence of nucleic acid monomers complementary to the sequence of a unique codon on at least one of the plurality of templates. In one aspect of the invention, building blocks are provided which possesses anti-codons complementing all the unique codons of the variety of templates. In another
15 aspect, some of the building blocks carrying anti-codons for selected unique codons are not present. The latter situation may occur when termination of the synthesis is desired at different stages or when the absences of certain functional entities on the templated molecule are desirable. Furthermore, anti-codons not associated with any functional entity may be present for steric reasons or to avoid unintended hybridisation events.

20 The design of the anti-codons and the complementing unique codons may be aimed at obtaining essentially the same annealing temperature for all or some of the codon:anti-codon hybrids to ensure that all the anti-codons have been annealed to the template before the functional entities are connected to
25 each other through a chemical reaction. In an aspect of the invention, the annealing temperature of the codon:anti-codon hybrids within the same coding region is designed to have different annealing temperature. The separate reaction may be accomplished by initially raising the temperature above the annealing temperature for all the hybrids and slowly decreasing the temperature
30 until the first set of anti-codons anneal to its templates. Following the connection of the functional entity to another functional entity or a nascent templated molecule, the temperature is decreased sufficient for allowing an-

other building blocks to anneal to a coding region in the vicinity. The above design provides for the possibility that functional groups of different templates but within the same coding region are subjected to different reaction conditions.

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A further design involves annealing temperatures different for each of the coding regions but similar within a specific coding region. Upon proper design it is possible step-wise to anneal the individual building blocks by gradually decreasing the temperature from above the annealing temperature for the total of the codon:anti-codon hybrids to a temperature at or below a temperature where all the codon:anti-codon hybrids have been formed within the same coding region. Subsequently or simultaneously with the temperature regime selected, a connection between the functional entities and the other functional entities or nascent templated molecules is performed. At each step of the step-wise decrease of the temperature, a new building block is annealed to the template and a subsequent connection is performed. This design allows for the simultaneous addition of all the building blocks to the plurality of templates and, thus, omitting the step-wise addition of building blocks.

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It is within the capability of the skilled person in the art to construct the desired design. When a specific annealing temperature is desired it is a standard procedure to suggest appropriate compositions of nucleic acid monomers and the length thereof. The construction of an appropriate design may be assisted by software, such as Vector NTI Suite or the public database at the internet address <http://www.nwfsc.noaa.gov/protocols/oligoTMcalc.html>.

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The functional entity of the building block serves the function of being a precursor for the structural entity eventually incorporated into the templated molecule.

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The functional entity is designed to be capable of being connected to a functional entity of another building block or a nascent templated molecule. The connection is aided by one or more reactive groups of the functional entity. The number of reactive groups which appear on the functional entity is suitably one to ten. A building block featuring only one reactive group is used *i.a.* in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. Two or more reactive groups intended for the formation of connections, are typically present on scaffolds. A scaffold is a core structure, which forms the basis for the creation of multiple variants. The variant forms of the scaffold is typically formed through reaction of reactive groups of the scaffold with reactive groups of other building blocks, optionally mediated by fill-in groups or catalysts, under the creation of a connection between the entities. The functional entities to be connected to the scaffold may contain one, two or several reactive groups able to form connections.

Some of the linkers are durable during the entire synthesis to ensure the connection between the scaffolded molecule and the template. It is essential for the invention that at least one linker is maintained in order to ensure the coupling of the templated molecule to the template which directed the synthesis thereof. In a final state of the production of the templated molecule the at least one linker emanating from a building block may be substituted by another entity securing the adherence of the template molecule to the template. The durable linkers are preferably cleavable at a final stage to separate the templated molecule from the template or a complementary template. Therefore, the durable linkers may be referred to as selectively cleavable linkers. Preferably, some of the linkers of the building blocks are also cleavable during the synthesis of the templated molecule, *i.e.* the functional entity may be released from the building block to allow the synthesis of the templated molecule.

The linker may be attached to the anti-codon at a central area thereof or at one of the ends. In one aspect of the invention, the anti-codon and the linker is a contiguous oligonucleotide, *i.e.* a part of the nucleotide complements a sequence of the template and another part is non-complementing avoiding the hybridisation of the oligonucleotide part to the template. This design of the building blocks is a convenient way of design as no separate reaction step is required for the attachment of the linker to the anti-codon. In another aspect of the invention the linker is attached to a central part of the anti-codon to allow for the ligation of neighbouring anti-codons using suitable enzymes to produce a complementary template.

The linker can be attached to the functional entity according to the functionalities desired. In one aspect, the linker is attached to the functional entity through a reactive group capable of forming a connection to another functional entity or a nascent templated molecule. Examples of suitable reactive groups are imine groups (-NH-) and disulfide groups (-S-S-). The bonding of the functional entity to the linker can be cleaved simultaneously with the reaction of the inter-spacing reactive group or the cleavage can be performed in a separate step. In the following, linkers connected to a functional entity through a reactive group which is cleaved simultaneously with the formation of the connection, are referred to as translocating linkers. Translocating linkers allow for the production of templated polymers, which are connected to the template that directed the synthesis thereof via the terminal building block, when a reactive group on a functional entity in the vicinity reacts to form a connection. The separate formation of the connection between a functional entity and another functional entity or evolving templated molecule and the cleavage of the linker is an advantage because more than one connection may be formed prior to the cleavage.

A subset of the building blocks is contacted with the plurality of templates in the initial phase of the production of the library. The subset of the total amount of building blocks is selected to have anti-codons which complement

unique codons of a specific coding region on the template. It may be of advantage to have the building blocks in the vicinity of the reactive group of the template. In the event, the reactive group of the template is a part of a building block, it is preferred that the building blocks to be linked together is attached in the vicinity of each other to ensure a sufficient proximity of the functional entities. Preferably, the subset comprises building blocks having anti-codons which form hybrids with unique codons in two neighbouring coding regions. The subset may be provided by adding the building blocks separately, or alternatively, by adding all the building blocks or a major portion thereof and then direct the annealing of the individual building blocks by proper design of the codon:anti-codon hybrids, as depicted above.

The conditions which allow specific hybridisation of the unique codons and the anti-codons are influenced by a number of factors including temperature, salt concentration, type of puffer, and acidity. It is within the capabilities of the person skilled in the art to select appropriate conditions to ensure that the contacting between the templates and the building blocks are performed at hybridisation conditions.

When two building blocks in the initial stage of the present method are hybridised to a template the functional entities of each of the building blocks are allowed to form a chemical connection. The connection between two functional entities usually occurs by a reaction between reactive groups present on each functional entity. It may, however, be desirable to make the bridging between two reactive groups on separate functional entities through a suitable fill-in group. The latter situation may occur, for example, when two similar reactive groups, such as two amine groups, are not able to react with each other directly. The two amine groups may, however, be connected to each other through a di-carboxylic acid, such as oxalic acid, to create amide bondings.

Following the successful connection of two entities for the formation of the nascent templated compound, one of the linkers connected thereto may be cleaved. However, this step is optional. It is possible to continue with the incorporation of further building blocks without this cleaving step. In one aspect of the invention, the connection of the functional entities and the cleavage of one of the linkers occur simultaneously. This aspect of the invention is of particular relevance when the reactive group able to react with another reactive group of a functional entity in the vicinity thereof, is the bridging group to the linker.

The propagation part of the method is initiated by contacting the plurality of different templates harbouring the nascent templated compound with a further subset of the plurality of building blocks, said subset having anti-codons complementary to the unique codons of a coding region in the vicinity of the coding region(s) harbouring the nascent templated molecules. To increase the proximity, it can be suitable to hybridise the further building blocks to a coding region neighbouring the building block(s) harbouring the nascent templated molecule. The subset of building blocks may be provided by adding building blocks separately which hybridise to a specific coding region. Alternatively, different stringency conditions combined with appropriate design of the individual codon:anti-codon hybrids can be selected to allow the predominate annealing of a subset of building blocks to selected codon regions. The alternative way of forming a subset has the advantage that all or at least a major part of the building blocks can be added to the reaction vessel. An example of directing the annealing is to design the first two coding regions such that they have a relatively high annealing temperature, whereas the subsequent building blocks to be annealed have a gradually decreasing annealing temperature.

When the further building block is hybridised to a template also harbouring the evolving templated molecule, the functional entity of the further building block is allowed to form a chemical connection to the nascent templated

- molecule. The formation of the chemical connection normally proceeds by reaction between reactive groups present on the functional entity and the nascent templated molecule, respectively. It may, however, be desirable to make the bridging between two reactive groups through a suitable spacer group. The latter situation may for example occur when two similar reactive groups, such as a two amine groups, are not able to react with each other directly. The two amine groups may, however, be connected to each other through a di-carboxylic acid, such as oxalic acid, to create amide bondings.
- After the connection between the nascent templated molecule and the further functional entity one or more of the linkers may optionally be cleaved, however ensuring that at least one linker is durable. The propagation part of the method may be repeated a desired number of times to evolve the templated molecule. Each repetition of the propagation is initiated by contacting the templated with a new subset of further building blocks.

- After the propagation stage follows the termination stage. Depending on the degree of cleavage during the propagation, the attachment of the templated molecule or the complementary template, is different. At one extreme none of the linkers are cleaved during the synthesis, which is when the optional cleavage of linkers of step g) is not performed. This may lead to a templated molecule attached to a number of linker similar to the number of building blocks involved in the synthesis. At the other extreme, the complexes obtained in step i) comprise templated molecules attached to the template which templated the syntheses thereof via the linker of a single building block. The number of linkers may be anywhere between these two extremes in the templated molecule. In some applications it is of advantage to have more than one linker, e.g. when the conformation of the templated molecule can be stabilized or even altered. In general, it is sufficient for the templated molecule to be attached to the template through a single linker to allow the subsequent enrichment process to proceed efficiently.

The attachment via a building block involves the use of hydrogen bondings between the codon and the anti-codon to ensure the coupling of the template and the templated molecule. As is well-known within the art, hydrogen bondings are weak bondings that may easily be disrupted. Therefore, in an aspect of the invention, the building block finally harbouring the templated molecule, may be attached to the template through a codon:anti-codon hybrid having a higher annealing temperature than the other codon:anti-codon hybrids of the template. Alternatively, and in some applications preferably, the templated molecule is connected with the template which directed the syntheses thereof via a covalent link. The covalent link may be in addition to the hydrogen bondings or the covalent link may be a substitution. The presence of a covalent link allows for a more harsh chemical treatment of the complex. In one aspect of the invention, the covalent link is selectively cleavable to provide for a separation of the templated molecule from the complementary template.

The method according to the present invention may involve the further step of cleaving all but one linker after the formation of the templated molecule. This further step implies that some or all of the linkers in step g) are not cleaved during the synthesis of the templated molecule.

The method according to the invention may, as a further step, involve the transfer of the templated molecule to an anchorage point on the template, or a sequence complementing the template, to establish an effective chemical connection between the template and the templated molecule. An effective coupling of the templated molecule to the template or a sequence complementary to the template can be desirable to allow for denaturing enrichment conditions or denaturing post-templating modification of the manufactured molecule. The anchorage may involve the presence of a reactive group on the templated molecule and a reaction partner on the template, whereby the reaction between these reactive groups will establish a covalent link. Alternatively, the anchorage point may be present on a complementary sequence hybridised to the template. In a preferred embodiment the complementing

sequence has a higher annealing temperature than one or more of the building blocks, notably the terminal building block, to enable usage of a higher stringency during enrichment and , optionally, clearance of used building blocks.

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The anti-codons can, after the cleavage of the linker, remain hybridised to the unique codons or can be detached from the template. When it is chosen to detach the anti-codons, they are preferably cleared from the solution to avoid any re-hybridisation or interference with nucleic acids intended to participate in an hybridisation event. In an aspect of the invention, the anti-codons following the cleavage of the linker attached thereto, remain hybridised to the unique codons because the anti-codons attached to the templates can be ligated together to create a complementary template. The ligation of the anti-codons may be performed after all or the majority of building blocks have been incorporated or, in the alternative, the ligation can be performed after the incorporation of each new building block. Furthermore, in some occasions, it may be of advantage to ligate the anti-codons together prior to the cleavage of the linkers. The ligation of the all the anti-codons provide a direct covalent link between the complementing template and the templated molecule. The covalent link is preferably designed to allow the separation of the templated molecule from the complementing template. The separation of the templated molecule will in general during an enrichment process appear as one of the later steps. Therefore, it is of importance in most applications that the covalent link is selectively cleavable, i.e. cleavable under certain chemical conditions not used in the prior steps of enrichment. In one aspect of the invention the templated molecule is released by the use of enzymes. As an example, restriction nucleases may be used by the incorporation of a restriction site close to the templated molecule. Another example is to use a phosphodiesterase to perform a total or partly digest of the template or complementing template.

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The use of ligation also have another advantage, because it is possible to use anti-codons having a shorter sequence of nucleotides. An example of a typically anti-codon will have 15 to 25 nucleotides in sequence to obtain an appropriate annealing temperature of around 40 to 70°C. By ligating building blocks in the vicinity of each other using a ligase or a chemical crosslink, it becomes possible to incorporate very small anti-codons (e.g. 4-10 nucleotides) with high specificity and efficiency. The ligation of a small anti-codon to another anti-codon or a complementing template increases the total annealing temperature. A result of using smaller anti-codons is that, the local concentration of functional entities is increased, and therefore, the efficiency of the reaction between the reactive groups becomes more efficient.

Another way to increase the proximity further is to provide building blocks intended to interact with each other, with a reversible interacting molecule pair. The molecule pair allows a building block to form a reversible coupling to another building block in the vicinity thereof through the interaction of the two parts of the pair situated on each building block. Preferably, the molecule pair is also termed a dimerisation domain and is located in the functional entity or is a portion of the linker that is close to the functional entity. The dimerisation domains of two building blocks intended to react with each other are designed to have an affinity to each other. Examples of dimerisation domains include leucine-rich areas, coiled-coil structures, antibody-antigen pairs, complementing sequences of nucleotides, ect. The affinity of the dimerisation of two building blocks are preferably lower than the affinity between the codon and the anti-codon to allow for shifting dimerisation partners though performing temperature cycles. When the dimerisation domains of two building blocks intended to interact are sequences of nucleotides, the length of the sequences may be chosen to obtain an annealing temperature below room temperature but preferably above 5°C, e.g. between 10°C and 20°C. When the dimerisation domain includes two complementing oligonucleotides, the domain is also referred to as a "zipper box".

A suitable temperature scheme for the propagation step of the present invention is to add a building block to the template at a temperature above the annealing temperature for said building block. Then the temperature is lowered below the annealing temperature to allow the new building block as well as the building block harbouring the nascent templated molecule to find and bind to their respective parts of the template. Excess building blocks and debris are then preferably washed away. Then the temperature is decreased below the annealing temperature of the dimerisation domain and consequently the probability that the functional entities are connected is increased. Potentially, the conditions in the reaction vessel, other than the temperature, may be changed to provide for the connection. Following the connection between the functional entities/templated molecules, the temperature is raised and the temperature scheme is repeated.

Another method of increasing the proximity further is to apply a "rigid" linker attached to the anti-codon with molecular hinges. A result of using a rigid linker is that a smaller three dimensional space is sampled by the functional entity. Thereby the probability is increased that two functional entities in the vicinity of each other and attached to a rigid linker will be close enough to each other for a reaction to proceed. A rigid linker may be prepared by various methods available to the skilled person in the art. An example is to use a double stranded oligonucleotide. In a preferred embodiment, the anti-codon and the linker is performed of a contiguous oligonucleotide, wherein the anti-codon domain of the oligonucleotide is able to hybridise to a unique codon of a template and a stiffer domain is able to hybridise to a further oligonucleotide complementary thereto. The hinges may be provided by any group allowing essentially unhindered rotation about at least one bonding. A hinge may be provided in the above preferred embodiment by separation of the anti-codon domain and the stiffer domain with at least one nucleotide. In other words, a hinge may be provided by a single stranded region positioned between the double stranded unique codon:anti-codon hybrid and the double stranded rigid linker.

In one aspect of the invention, the template is covalently connected to the complementing template. The connection may be performed by covalently bonding the two hybridised strings to each other. In the alternative, the template may at one end be designed with a hair pin loop to enable the ligation of the template end to an anti-codon. According to this aspect, the templated molecule will be linked to a double stranded template. The double stranded template may be an advantage because it is more stable allowing more versatile chemical reactions.

In one aspect of the invention a library of complexes is obtainable from the above methods.

The library may be used for a variety of applications, including the search for compounds for use in therapeutic or diagnostic methods and plant protection compounds, like pesticides, fungicides ect. The library may comprise any number of complexes according to the invention. At one extreme, the library consists of only two complexes. At the other extreme, the library can consist of up to 10^{18} complexes. Usually, the number of complexes is to be selected between these two extremes.

One method to identify the most active compounds which can be used in e.g. therapeutic applications is to subject the library to an enrichment treatment. According to one aspect of the invention an enrichment of a library of complexes comprising templated molecules with respect to a predetermined activity, comprises the steps of:

- i) establishing a first library of complexes comprising templated molecules, said library being obtainable according to any of the methods of the invention,
- ii) exposing the library to conditions enriching the library with complexes having the predetermined activity,
- iii) amplifying the complexes of the enriched library,

- iv) optionally, repeating step ii) to iii), and
- v) obtaining an enriched library having a higher ratio of complexes comprising templated molecules with the predetermined activity.

5 The amplification is normally preferred, though not always necessary. Especially, when several cycles of enrichments are conducted it is of advantage to make an amplification to obtain sufficient complexes. In a preferred aspect of the invention, the amplification of the complexes of the enriched library comprises the steps of contacting the library of complexes
10 with amplification means, amplifying the templates or the complementing templates, and conducting the method according to the invention using the amplification product as templates. The amplification means can be any of the nucleic acid amplification means suitable for the amplification of the template, such as PCR. Preferably, the amplification of the complex
15 comprises a 10^1 to 10^{15} -fold amplification.

To allow for multiple enrichment cycles the steps ii) and iii) are repeated at least 2, 3, 5 times, such as at least 10 times, such as at least 15 times. The complexes may be identified after the completion of each cycle or may be
20 only be identified after the last cycle. There is no explicit need for intermediate identifications as the amplification can be performed without knowing the sequence of the template or a sequence complementing the template, if the template or the complement thereof is provided with suitable primer regions. The identification after the enrichment process involves the
25 determination of the sequence of the template and/or the structural determination of the templated molecule and/or the entire complex having the predetermined activity.

Preferably, the conditions enriching the library comprise contacting a binding
30 partner to the templated molecules of interest. The binding partner may be in solution or may be directly or indirectly immobilised on a support. The enrichment is in general performed using an affinity or activity assay. In one

aspect of the invention, the enrichment is conducted by screening for complexes having an affinity for – or an effect on – a target molecule or a target entity. In another aspect the enrichment is conducted by selection for catalytic activity. Alternatively, the conditions enriching the library involves
5 any one or more of electrophoretic separation, gelfiltration, immunoprecipitation, isoelectric focusing, centrifugation, and immobilization.

The enrichment process can involve cells. Thus, in one embodiment, the conditions enriching the library comprises providing cells capable of
10 internalising the templated molecule, or performing an interaction with the templated molecule having the desired predetermined activity.

When the library of complexes have been enriched to a small pool comprising complexes displaying a predetermined activity, it is desirable to obtain
15 each of the complexes separately. Thus, the invention also entails to a method for the manufacture of a complex of a templated molecule attached to the template which directed the synthesis thereof, said method comprises the steps of

- a) providing a template comprising a number of coding regions and a reactive group, wherein each coding region specifies a unique codon,
20
- b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of each building block complements a unique codon of the template, and
25 the functional entity comprises at least one reactive group,
- c) contacting the template with a building block having an anti-codon which complements the unique codon of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codon to the unique codon of the templates,
30
- d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,

- 5 e) contacting under conditions allowing specific hybridisation, the template harbouring the nascent templated molecule with a further building block having an anti-codon complementary to the unique codon of a coding region in the vicinity of the coding region harbouring the nascent templated molecule,
- f) allowing the functional entity of the further building block to form a chemical connection to the nascent templated molecule,
- 10 g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,
- h) optionally repeating steps e) through g),
- i) obtaining a templated molecule attached via the linker of one or more building blocks to the template which directed the synthesis thereof.

15 The templated molecule can be obtained from the complex by cleaving the linker(s) of the one or more building blocks to release the templated molecule from the template.

20 Detailed disclosure of the invention

Codon

The codons occurring in nature consist of a sequence of three nucleic acid monomers. The length of the codon sequence may be of the same order in accordance with the present invention but is preferably longer to obtain a suitable annealing temperature. Preferably the sequence is selected to produce an annealing temperature above normal room temperature. Herein the terms annealing temperature and melting temperature may be used interchangeably said temperature being defined as the maximum of the first derivative of the absorbance vs. temperature curve. The different codons need no to be of the same lengths, that is to comprise the same number of nucleic acid monomers. However, a typically number of nucleic acid monomers in the codon sequence is normally above 6 but below 25.

25

30

Each nucleic acid monomer is normally composed of three parts, namely a nucleobase moiety, a sugar moiety and a internucleoside linker.

5 The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. It should be clear to the person skilled in the art that various nucleobases which previously have been considered "nonnaturally occurring" have subsequently been found in nature. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof.
10 Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N⁶-methyladenine, 7-deazaxanthine, 7-deazaguanine, N⁴,N⁴-ethanocytosin, N⁶,N⁶-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C³-C⁶)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover every and all of these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine,
20 thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

The sugar moiety is suitably a pentose but may be the appropriate part of an PNA. Suitable examples of possible pentoses include ribose, 2'-deoxyribose,
25 2'-O-methyl-ribose, 2'-fluor-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity. An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer. The internucleoside linkage may be the natural occurring phosphodiester linkage or a derivative thereof. Examples of such
30 derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the internucleoside

linker can be any of a number of non-phosphorous-containing linkers known in the art.

5 Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine.

10 Each codon is complemented by an anti-codon. The anticodon has the ability specifically to engage with the codon which it complements. The affinity between the codon and the complementing anti-codon is affected through hydrogen bondings following the well-known Watson-Crick base pairing system.
15 Thus, the anti-codon may be composed of the same kind of nucleic acid monomers as the codon itself.

Linkers

20 Linkers connecting the anti-codon and functional entity of building blocks may be selected from a variety of possibilities. Linkers may include one or more reactive groups in order to obtain a selectively cleavable linker, a cleavable linker, and a translocating linkers. Suitable linkers may be selected from but are not limited to, the group comprising: carbohydrides and substituted carbohydrides; vinyl, polyvinyl and substituted polyvinyl; acetylene, polyacetylene; aryl/hetaryl, polyaryl/hetaryl and substituted polyaryl/polyhetaryl; ethers, 25 polyethers such as e.g. polyethylenglycol and substituted polyethers; amines, polyamines and substituted polyamines; double stranded, single stranded or partially double stranded natural and unnatural polynucleotides and substituted double stranded, single stranded or partially double stranded 30 natural and unnatural polynucleotides; and polyamides and natural and un-

natural polypeptides and substituted polyamides and natural and unnatural polypeptides.

5

Functional groups

The functional entity may comprise one or more functional groups, i.e. groups which eventually form part of the templated molecule. The templated molecule may comprise one or more of the following functional groups either alone or in combination:

10

1. Hydroxyls
2. Primary, secondary, tertiary amines
3. Carboxylic acids
4. Phosphates, phosphonates
- 15 5. Sulfonates, sulfonamides
6. Amides
7. Carbamates
8. Carbonates
9. Ureas
- 20 10. Alkanes, Alkenes, Alkynes
11. Anhydrides
12. Ketones
13. Aldehydes
14. Nitratates, nitrites
- 25 15. Imines
16. Phenyl and other aromatic groups
17. Pyridines, pyrimidines, purines, indole, imidazole, and heterocyclic bases
18. Heterocycles
- 30 19. polycycles
20. Flavins
21. Halides

- 22. Metals
- 23. Chelates
- 24. Mechanism based inhibitors
- 25. Small molecule catalysts
- 5 26. Dextrins, saccharides
- 27. Fluorescein, Rhodamine and other fluorophores
- 28. Polyketides, peptides, various polymers
- 29. Enzymes and ribozymes and other biological catalysts
- 30. Functional groups for post-polymerization/post activation coupling of
- 10 functional groups
- 31. Drugs, e.g., taxol moiety, acyclovir moiety, "natural products"
- 32. Supramolecular structures, e.g. nanoclusters
- 33. Lipids
- 34. Oligonucleotides, oligonucleotide analogs (e.g., PNA, LNA, mor-
- 15 pholinos)
- 35. Hydrogen

Reactive groups

20 Reactive groups relates among other things to groups which form part of the functional entity and are capable of participating in a reaction that form a connection between two functional entities, either directly or via a suitable bridging molecular entity. Examples of reactive groups are listed below:

- 1. N-carboxyanhydrides (NCA)
- 2. N-thiocarboxyanhydrides (NTA)
- 25 3. Amines
- 4. Carboxylic acids
- 5. Ketones
- 6. Aldehydes
- 7. Hydroxyls
- 30 8. Thiols
- 9. Esters
- 10. Thioesters

11. conjugated system of double bonds
12. Alkyl halides
13. Hydrazines
14. N-hydroxysuccinimide esters
5 15. Epoxides
16. Haloacetyls
17. UDP-activated saccharides
18. Sulfides
19. Cyanates
10 20. Carbonylimidazole
21. Thiazinanones
22. Phosphines
23. Hydroxylamines
24. Sulfonates
15 25. Activated nucleotides
26. Vinylchloride
27. Alkenes, quinones

20 Templated molecules

According to the present invention, virtually any molecule may be templated using the general method disclosed herein. Examples of compounds which it is anticipated can be synthesised includes, but are not limited to, the compounds listed below:

25

alpha-, beta-, gamma-, and omega-peptides Mono-, di- and tri-substituted peptides; L- and D-form peptides; Cyclohexane- and cyclopentane-backbone modified beta-peptides; Vinylogous polypeptides; glycopolypeptides; polyamides; vinylogous sulfonamide peptide; polysulfonamide; conjugated peptide
30 (i.e., having prosthetic groups); polyesters; polysaccharides; polycarbamates; polycarbonates; polyureas; poly-peptidylphosphonates; azatides; peptoids (oligo N-substituted glycines); polyethers; ethoxyformacetal oligomers; poly-

thioethers; polyethylene glycols (PEG); polyethylenes; polydisulfides; polyarylene sulfides; polynucleotides; PNAs; LNAs; morpholinos; oligo pyrrolidone; polyoximes; polyimines; polyethyleneimine; polyacetates; polystyrenes; polyacetylene; polyvinyl; lipids; phospholipids; glycolipids; polycycles (aliphatic); polycycles (aromatic); polyheterocycles; proteoglycan; polysiloxanes; polyisocyanides; polyisocyanates; polymethacrylates; monofunctional, Difunctional, Trifunctional and Oligofunctional open-chain hydrocarbons; monofunctional, difunctional, trifunctional and oligofunctional nonaromatic carbocycles; monocyclic, bicyclic, tricyclic and polycyclic hydrocarbons; bridged polycyclic hydrocarbons; monofunctional, difunctional, trifunctional, and oligofunctional nonaromatic heterocycles; monocyclic, bicyclic, tricyclic, and polycyclic heterocycles, bridged polycyclic heterocycles; monofunctional, difunctional, trifunctional and oligofunctional aromatic carbocycles; monocyclic, bicyclic, tricyclic, and polycyclic aromatic carbocycles; monofunctional, difunctional, trifunctional and oligofunctional aromatic heterocycles; monocyclic, bicyclic, tricyclic and polycyclic heterocycles; chelates; fullerenes; steroids; cyclosporin analogs; as well as any combination of the above molecular moieties.

20 Use of Library

Selection or screening, commonly referred to as enrichment, of the library of complexes comprising templated molecules with respect to desired activities (for example binding to particular target, catalytic activity, or a particular effect in an activity assay) may be performed according to any standard protocol. For example, affinity selections may be performed according to the principles used for phage displayed, polysome-displayed or mRNA-protein fusion displayed peptides. Selection for catalytic activity may be performed by affinity selections on transition-state analogue affinity columns (Baca et al., Proc. Natl. Acad. Sci USA. 1997; 94(19):10063-8), or by function-based selection schemes (Pedersen et al., Proc. Natl. Acad. Sci. USA. 1998, 95(18):10523-8). Screening for a desired characteristic may be performed according to standard microtiter plate-based assays, or by FACS-sorting assays.

- Generally, affinity selections involve the immobilisation of a target or a binding partner on a solid support, such as a column. Subsequently, the complex manufactured according to the invention is added to the column under conditions allowing a part of the complexes to bind to the target. The complexes not bound to the target is eluted out of the column and discharged. The part of the complexes attached to the target may be amplified using the template or complementing template associated with the templated molecule.
- 10 The choice of amplification method depends on the choice of codons and anti-codons. Natural oligonucleotides can be amplified by any state of the art method. These methods include, but is not limited to the polymerase chain reaction (PCR); as well as e.g. nucleic acid sequence-based amplification (e.g. Compton, Nature 350, 91-92 (1991)), amplified anti-sense RNA (e.g. 15 van Gelder et al., PNAS 85: 77652-77656 (1988)); self-sustained sequence replication system (e.g. Gnatelli et al., PNAS 87: 1874-1878 (1990)); polymerase independent amplification as described in e.g. Schmidt et al., NAR 25: 4797-4802 (1997), as well as in vivo amplification of plasmids carrying cloned DNA fragments. Ligase-mediated amplification methods may 20 also be used, e.g., LCR (Ligase Chain Reaction).

For non-natural nucleotides the choices of efficient amplification procedures are fewer. As non-natural nucleotides per definition can be incorporated by certain enzymes including polymerases, it will be possible to perform manual 25 polymerase chain reaction by adding the polymerase during each extension cycle.

For oligonucleotides containing nucleotide analogs, fewer methods for amplification exist. One may use non-enzyme mediated amplification 30 schemes (Schmidt et al., NAR 25: 4797-4802 (1997)). For backbone-modified oligonucleotide analogs such as PNA and LNA, this amplification method may be used. Before or during amplification the templates or

complementing templates may be mutagenized or recombined in order to create a larger diversity for the next round of selection or screening.

5 Following the amplification of the template part or complementing template part of the complex, the method according to the invention is conducted using the amplification product as the templates. The result is a reduced or enriched library of complexes of a template attached to a template molecule.

10 The selection and amplification steps may be repeated if considered necessary to further enrich the library. When the selection and amplification steps are repeated, the binding step involving the target and the complexes, is preferably performed under more strict conditions ensuring that only a part of the complexes adhere to the target.

15 The enrichment cycles may be performed 2 to 15 times or even more with enrichment in each cycle of 10 to 1000 times. In one approach, the starting library amounts to 10^{14} complexes. After seven cycles of enrichments with a 100 fold concentration in each cycle, the complex with the highest affinity to the target should, theoretically, be obtained. However, it is more likely that
20 the final cycles deliver a small pool of interesting complexes, which have to be examined by other means.

25 After the final round of selection, it is often desirable to sequence individual templates, in order to determine the composition of individual templated molecules. If the template contains natural nucleotides, it is a standard routine to optionally PCR amplify the isolated templates (if the template is an RNA molecule, it is necessary to use reverse transcriptase to produce cDNA prior to the PCR-amplification), and then clone the DNA fragments into for example plasmids, transform these and then sequence individual plasmid-
30 clones containing one or multiple tandem DNA sequences. In this case, it is practical to design a restriction site in both of the flanking sequences to the central random or partly random sequence of the template (i.e., in the primer

binding sites). This will allow easy cloning of the isolated nucleotides. Sequencing can be done by the standard dideoxy chain termination method, or by more classical means such as Maxam-Gilbert sequencing.

- 5 If the template contains non-natural nucleotides, it may not be feasible to clone individual sequences by transfer through a microbial host. However, using bead populations where each bead carries one oligonucleotide sequence, it is possible to clone in vitro, where after all the nucleotides attached to a specific bead may be optionally amplified and then sequenced (Brenner
10 et al., 2000, Proc. Natl. Acad. Sci. USA 97, 1665-1670). Alternatively, one may dilute the population of isolates adequately, and then aliquot into microtiter plates so that the wells on average contain for example 0.1 templates. By amplifying the single templates by for example PCR, it will now be possible to sequence using standard methods. Of course, this requires that the
15 non-natural nucleotides are substrates for the thermostable polymerase used in the PCR.

- If alternative methods are used that require shorter oligonucleotides it may be desirable to design the starting template so as to contain restriction sites on
20 either side of the encoding/templating region of the template. Thereby, after the final selection round, the templates can be restricted, to obtain a short oligonucleotide encoding the templated polymer, and then these short oligonucleotides can be applied to various analytical procedures.

- 25 It is also possible to sequence the isolates by the use of a DNA array of oligonucleotides with random but predetermined sequences.

- It may also be desirable to sequence the population of isolates as a pool, for example if the sequences are expected to be in register, for example because the initial library consisted of a degenerate sequence based on a
30 polymer sequence with a known (relatively high) desired activity. Therefore, it is then expected that all the isolates have sequences similar to the initial

sequence of the templates before selection. Thus, the population of isolates can be sequenced as a whole, to obtain a consensus sequence for the population as a whole.

5 *Selection of template-displaying molecules that will bind to known targets*
The present invention is also directed to approaches that allow selection of small molecules capable of binding to different targets. The template-
displaying molecule technology contains a built-in function for direct selection
and amplification. The binding of the selected molecule should be selective in
10 that they only coordinate to a specific target and thereby prevent or induce a specific biological effect. Ultimately, these binding molecules should be possible to use e.g. as therapeutic agents, or as diagnostic agents.

Template-displaying molecule libraries can easily be combined with screen-
15 ings, selections, or assays to assess the effect of binding of a molecule ligand on the function of the target. In a more specific embodiment, the template-displaying method provides a rapid means for isolating and identifying molecule ligands which bind to supra-molecular, macro-supra-molecular, macro-molecular and low-molecular structures (e.g. nucleic acids and pro-
20 teins, including enzymes, receptors, antibodies, and glycoproteins); signal molecules (e.g. cAMP, inositol triphosphate, peptides, prostaglandins); and surfaces (e.g. metal, plastic, composite, glass, ceramics, rubber, skin, tissue).

25 Specifically, selection or partitioning in this context means any process whereby the template-displaying molecule complex bound to a target molecule, the complex-target pair, can be separated from template-displaying molecules not bound to the target molecule. Selection can be accomplished by various methods known in the art.

30 The selection strategy can be carried out so it allows selection against almost any target. Importantly, no steps in this selection strategy need any detailed

structural information of the target or the molecules in the libraries. The entire process is driven by the binding affinity involved in the specific recognition/coordination of the molecules in the library to a given target. However, in some applications, if needed, functionality can also be included analogous to selection for catalytic activity using phage display (Soumillion et al. (1994) J. Mol. Biol. 237: 415-22; Pedersen et al. (1998) PNAS. 18: 10523-10528). Example of various selection procedures are described below.

This built-in template-displaying molecule selection process is well suited for optimizations, where the selection steps are made in series starting with the selection of binding molecules and ends with the optimized binding molecule. The single procedures in each step are possible to automate using various robotic systems. This is because there is a sequential flow of events and where each event can be performed separately. In a most preferable setting, a suitable template-displaying molecule library and the target molecule are supplied to a fully automatic system which finally generates the optimized binding molecule. Even more preferably, this process should run without any need of external work outside the robotic system during the entire procedure.

The libraries of template-displayed molecules will contain molecules that could potentially coordinate to any known or unknown target. The region of binding on a target could be into a catalytic site of an enzyme, a binding pocket on a receptor (e.g. GPCR), a protein surface area involved in protein-protein interaction (especially a hot-spot region), and a specific site on DNA (e.g. the major groove). The template-displaying molecule technology will primarily identify molecules that coordinate to the target molecule. The natural function of the target could either be stimulated (agonized) or reduced (antagonized) or be unaffected by the binding of the template-displaying molecules. This will be dependent on the precise binding mode and the particular binding-site the template-displaying molecules occupy on the target. However, it is known that functional sites (e.g. protein-protein interaction or catalytic sites) on different proteins are more prone to bind molecules that

other more neutral surface areas on a protein. In addition, these functional sites normally contain a smaller region that seems to be primarily responsible for the binding energy, the so called hot-spot regions (Wells, et al. (1993) Recent Prog. Hormone Res. 48; 253-262). This phenomenon will increase
5 the possibility to directly select for small molecules that will affect the biological function of a certain target.

The template-displaying molecule technology of the invention will permit selection procedures analogous to other display methods such as phage display (Smith (1985) Science 228: 1315-1317). Phage display selection has
10 been used successfully on peptides (Wells & Lowman. (1992) Curr. Op. Struct. Biol. 2, 597-604) proteins (Marks et al. (1992) J. Biol. Chem. 267: 16007-16010) and antibodies (Winter et al. (1994) Annu. Rev. Immunol. 12: 433-455). Similar selection procedures are also exploited for other types of
15 display systems such as ribosome display (Mattheakis et al. (1994) Proc. Natl. Acad. Sci. 91: 9022-9026) and mRNA display (Roberts, et al. (1997) Proc. Natl. Acad. Sci. 94: 12297-302). However, the template-displaying molecule technology of the invention, will for the first time allow direct selection of target-specific small non-peptide molecules independently of the
20 translation process on the ribosome complex. The necessary steps included in this invention are the amplification of the templates and incorporation and reaction of the monomer building blocks. The amplification and incorporation and the incorporation and reaction are either done in the same step or in a sequential process.

25 The linkage between the templated molecule (displayed molecule) and DNA replication unit (coding template) allows a rapid identification of binding molecules using various selection strategies. This invention allows a broad strategy in identifying binding molecules against any known target. In addition,
30 this technology will also allow discovery of novel unknown targets by isolating binding molecules against unknown antigens (epitopes) and use these bind-

ing molecules for identification and validation (see section "*Target identification and validation*").

As will be understood, selection of binding molecules from the template-
5 displaying molecule libraries can be performed in any format to identify optimal binding molecules. A typical selection procedure against a purified target will include the following major steps: Generation of a template-displaying molecule library; Immobilization of the target molecule using a suitable immobilization approach; Adding the library to allow binding of the template-
10 displayed molecules; Removing of the non-binding template-displayed molecules; Elution of the template-displayed molecules bound to the immobilized target; Amplification of enriched template-displaying molecules for identification by sequencing or to input for the next round of selection. The general steps are schematically shown in Figure 27.

15 In a preferred embodiment, a standard selection protocol using a template-displaying molecule library is to use the bio-panning method. In this technique, the target (e.g. protein or peptide conjugate) is immobilized onto a solid support and the template-displayed molecules that potentially coordinate to the target are the ones that are selected and enriched. However, the
20 selection procedure requires that the bound template-displayed molecules can be separated from the unbound ones, i.e. those in solution. There are many ways in which this might be accomplished as known to ordinary skilled in the art.

25 The first step in the affinity enrichment cycle (one round as described in Figure 27) is when the template-displayed molecules showing low affinity for an immobilized target are washed away, leaving the strongly binding template-displayed molecules attached to the target. The enriched population, remaining bound to the target after the stringent washing, is then eluted with, e.g.
30 acid, chaotropic salts, heat, competitive elution with the known ligand or proteolytic release of the target/template molecules. The eluted template-

displayed molecules are suitable for PCR, leading to many orders of amplification, i.e. every single template-displayed molecule enriched in the first selection round participates in the further rounds of selection at a greatly increased copy number. After typically three to ten rounds of enrichment a population of molecules is obtained which is greatly enriched for the template-displayed molecules which bind most strongly to the target. This is followed quantitatively by assaying the proportion of template-displaying molecules which remain bound to the immobilized target. The variant template sequences are then individually sequenced.

Immobilisation of the target (peptide, protein, DNA or other antigen) on beads might be useful where there is doubt that the target will adsorb to the tube (e.g. unfolded targets eluted from SDS-PAGE gels). The derivatised beads can then be used to select from the template-displaying molecules, simply by sedimenting the beads in a bench centrifuge. Alternatively, the beads can be used to make an affinity column and the template-displaying libraries suspension recirculated through the column. There are many reactive matrices available for immobilizing the target molecule, including for instance attachment to -NH₂ groups and -SH groups. Magnetic beads are essentially a variant on the above; the target is attached to magnetic beads which are then used in the selection. Activated beads are available with attachment sites for -NH₂ or -COOH groups (which can be used for coupling). The target can be also be blotted onto nitrocellulose or PVDF. When using a blotting strategy, it is important to make sure the strip of blot used is blocked after immobilization of the target (e.g. with BSA or similar protein).

In another preferred embodiment, the selection or partitioning can also be performed using for example: Immunoprecipitation or indirect immunoprecipitation were the target molecule is captured together with template-displaying binding molecules; affinity column chromatography were the target is immobilized on a column and the template-displaying libraries are flowed through to capture target-binding molecules; gel-shift (agarose or polyacrylamide) were

the selected template-displaying molecules migrate together with the target in the gel; FACS sorting to localize cells that coordinates template-displaying molecules; CsCl gradient centrifugation to isolate the target molecule together template-displaying binding molecules; Mass spectroscopy to identify target molecules which are labelled with template-displaying molecules; etc., without limitation. In general, any method where the template-displaying molecule/target complex can be separated from template-displaying molecules not bound to the target is useful.

Table 1: Examples of selection method possible to use to identify binding molecules using the template-displaying technology.

Type of Target	Method of choice
Soluble receptors	Direct immobilization, Immunoprecipitation, affinity column, FACS sorting, MS.
Cell surface receptor	Cell-surface subtraction selection, FACS sorting, Affinity column.
Enzyme inhibitors	Direct immobilization, Immunoprecipitation, affinity column, FACS sorting, MS.
Surface epitopes	Cell-surface subtraction selection, in-vivo selection, FACS sorting, Affinity column.

Elution of template-displayed molecules can be performed in different ways. The binding molecules can be released from the target molecule by denaturation, acid, or chaotropic salts and then transferred to another vial for amplification. Alternatively, the elution can be more specific to reduce the background. Elution can be accomplished using proteolysis to cleave a linker between the target and the immobilizing surface or between the displaying molecule and the template. Also, elution can be accomplished by competition with a known ligand. Alternatively, the PCR reaction can be performed directly in the washed wells at the end of the selection reaction.

5 A possible feature of the invention is the fact that the binding molecules need not be elutable from the target to be selectable since only the encoding template DNA is needed for further amplification or cloning, not the binding molecule itself. It is known that some selection procedure can bind the most avid ligands so tightly as to be very difficult to elute. However the method of the invention can successfully be practiced to yield avid ligands, even covalent binding ligands.

10 Alternative selection protocol includes a known ligand as fragment of each displayed molecule in the library. That known ligand will guide the selection by coordinate to a defined part on the target molecule and focus the selection to molecules that binds to the same region. This could be especially useful for increasing the affinity for a ligand with a desired biological function but
15 with a too low potency.

A further aspect of the present invention relates to methods of increasing the diversity or complexity of a single or a mixture of selected binding molecules. After the initial selection, the enriched molecules can be altered to further increase the chemical diversity or complexity of the displayed molecules.
20 This can be performed using various methods known to the art. For example, using synthesized randomized oligonucleotides, spiked oligonucleotides or random mutagenesis. The randomization can be focused to allow preferable codons or localized to a predetermined portion or sub-sequence of the template nucleotide sequence. Other preferable method is to recombine templates coding for the binding molecules in a similar manner as DNA shuffling
25 is used on homologous genes for proteins (Stemmer (1994) Nature 370:389-91). This approach can be used to recombine initial libraries or more preferably to recombine enriched encoding templates.

30 In another embodiment of the invention when binding molecules against specific antigens that is only possible to express on a cell surface, e.g. ion channels or transmembrane receptors, is required, the cells particle themselves

can be used as the selection agent. In this sort of approach, cells lacking the specific target should be used to do one or more rounds of negative selection or be present in large excess in the selection process. Here, irrelevant template-displayed molecules are removed. For example, for a positive selection
5 against a receptor expressed on whole cells, the negative selection would be against the untransformed cells. This approach is also called subtraction selection and has successfully been used for phage display on antibody libraries (Hoogenboom et al. (1998) Immunotech. 4: 1-20).

10 A specific example of a selection procedure can involve selection against cell surface receptors that become internalized from the membrane so that the receptor together with the selected binding molecule can make its way into the cell cytoplasm or cell nucleus. Depending on the dissociation rate constant for specific selected binding molecules, these molecules largely reside
15 after uptake in either the cytoplasm or the nucleus.

The skilled person in the art will acknowledge that the selection process can be performed in any setup where the target is used as the bait onto which the template-displaying molecules can coordinate.

20

The selection methods of the present invention can be combined with secondary selection or screening to identify molecule ligands capable of modifying target molecule function upon binding. Thus, the methods described herein can be employed to isolate or produce binding molecules which bind to and
25 modify the function of any protein or nucleic acid. It is contemplated that the method of the present invention can be employed to identify, isolate or produce binding molecules which will affect catalytic activity of target enzymes, *i.e.*, inhibit catalysis or modifying substrate binding, affect the functionality of protein receptors, *i.e.*, inhibit binding to receptors or modify the specificity of
30 binding to receptors; affect the formation of protein multimers, *i.e.*, disrupt quaternary structure of protein subunits; and modify transport properties of protein, *i.e.*, disrupt transport of small molecules or ions by proteins.

A still further aspect of the present invention relates to methods allowing functionality in the selection process can also be included. For example, when enrichment against a certain target have been performed generation a
5 number of different hits, these hits can then directly be tested for functionality (e.g. cell signalling). This can for example be performed using fluorescence-activated cell sorting (FACS).

The altered phenotype may be detected in a wide variety of ways. Generally,
10 the changed phenotype is detected using, for example: microscopic analysis of cell morphology; standard cell viability assays, including both increased cell death and increased cell viability; standard labelling assays such as fluorometric indicator assays for the presence of level of particular cell or molecule, including FACS or other dye staining techniques; biochemical de-
15 tection of the expression of target compounds after killing the cells; etc. In some cases, specific signalling pathways can be probed using various reporter gene constructs.

Secondary selection methods that can be combined with template-displaying
20 molecule technology include among others selections or screens for enzyme inhibition, alteration or substrate binding, loss of functionality, disruption of structure, etc. Those of ordinary skill in the art are able to select among various alternatives of selection or screening methods that are compatible with the methods described herein.

25 The binding molecules of the invention can be selected for other properties in addition to binding, For example, during selection; stability to certain conditions of the desired working environment of the end product can be included as a selection criterion. If binding molecules which are stable in the presence
30 of a certain protease is desired, that protease can be part of the buffer medium used during selection. Similarly, the selection can also be performed in serum or cell extracts or any type of media. As will be understood, when util-

izing this template-displaying approach, conditions which disrupt or degrade the template should be avoided to allow amplification. Other desired properties can be incorporated, directly into the displaying molecules as will be understood by those skilled in the art. For example, membrane affinity can be
5 included as a property by employing building blocks with high hydrophobicity.

Molecules selected by the template-displaying molecule technology can be produced by various synthetic methods. Chemical synthesis can be accomplished since the structure of selected binding molecules is readily obtained
10 form the nucleic acid sequence of the coding template. Chemical synthesis of the selected molecules is also possible because the building blocks that compose the binding molecules are also known in addition to the chemical reactions that assemble them together.

15 In a preferred embodiment, the selected binding molecules is synthesized and tested in various appropriate *in vitro* and *in vivo* testing to verify the selected candidates for biological effects and potency. This may be done in a variety of ways, as will be appreciated by those in the art, and may depend on the composition of the bioactive molecule.

20

Target identification and validation

In another aspect, the present invention provides methods to identify or isolate targets that are involved in pathological processes or other biological events. In this aspect, the target molecules are again preferably proteins or
25 nucleic acids, but can also include, among others, carbohydrates and various molecules to which specific molecule ligand binding can be achieved. In principal, the template-displaying molecule technology could be used to select for specific epitopes on antigens found on cells, tissues or *in vivo*. These epitopes might belong to a target that is involved in important biological
30 events. In addition, these epitopes might also be involved in the biological function of the target.

Phage display with antibodies and peptide libraries has been used numerous times successfully in identifying new cellular antigens. (e.g. Pasqualini et al. (1996) Nature 380: 364-366; Pasqualini et al. (2000) Cancer Res. 60: 722-727; Scheffer et al. (2002) Br J Cancer 86: 954-962; Kupsch et al. (1999) Clin Cancer Res. 5: 925-931; Tseng-Law et al. (1999) Exp. Hematol. 27: 936-945; 5 Gevorkian et al. (1998) Clin. Immunol. Immunopathol. 86: 305-309). Especially effective have been selection directly on cells suspected to express cell-specific antigens. Importantly, when selecting for cell-surface antigen, the template molecule can be maintained outside the cell. This will increase the probability that the template molecule will be intact after release for the cell 10 surface.

In vivo selection of template-displayed molecules has tremendous potential. By selecting from libraries of template-displayed molecules in vivo it is possible to isolate molecules capable of homing specifically to normal tissues and 15 other pathological tissues (e.g. tumours). This principle has been illustrated using phage display of peptide libraries (Pasqualini & Ruoslathi (1996) Nature 280: 364-366). This system has also been used in humans to identify peptide motifs that localized to different organs (Arap et al. (2002) Nat. Med. 20 2:121-127). A similar selection procedure could be used for the template-displaying libraries. The coding DNA in phage display is protected effectively by the phage particle allows selection *in vivo*. Accordingly, the stability of the template *in vivo* will be important for amplification and identification. The template can be stabilised using various nucleotide derivatives in a similar 25 way as have been used to stabilise aptamers for in vivo applications (Nolte (1996) Nature Biotechnol. 14: 1116-1121; Págratis et al. (1997) Nature Biotechnol. 15: 68-72). However, it is reasonable to believe that the template structure will be stabilized against degradation due to the modified bases used for encoding the displayed molecule. Other types of protection are also 30 possible where the template molecule is shielded for the solution using various methods. This could include for example liposomes, pegylation, binding proteins or other sorts of protection. The template molecule could also be

integrated into another designed structure that protects the template from external manipulation. For example, the linker can be designed to be incorporated in vesicles to position the templates inside the vesicle and the displaying molecules on the outside. The arrangement will protect the template molecules from external manipulation and at the same time allow exposure of the displaying molecules to permit selection.

Most antibodies have a large concave binding area which requires to some degree protruding epitopes on the antigens. Also, the antibody molecule is a large macromolecule (150 KDa) which will sterically reduce the access for a number of different antigens (e.g. on a cell surface). The template-displaying technology should be able to access and recognize epitopes inaccessible to antibodies. The small binding molecules will be able to bind into active sites, grooves and other areas on an antigen. The coding template element is also smaller than an antibody which will increase the physical access of the template-binding molecule pair. In addition, the diversity and complexity of the template-displaying molecule libraries will be much greater compared to peptide libraries. This will increase the possibility to find molecules that can coordinate to epitopes inaccessible to peptides due to inadequate chemistry. All together, the template-displaying molecule technology has the potential to identify novel antigens which is not possible to identify with antibodies or peptides. One of ordinary skill in the art will acknowledge that various types of cells can be used in the selection procedure. It will also be understood that the selection for new antigens can be performed using subtraction methods as described previously.

Another aspect of the present invention relates to methods to validate the identified target. The identified binding molecules can directly be used if they change the biological response of the target. This can be done either *in vitro* using any direct or cell-based assay or directly *in vivo* studying any phenotypic response. The strength of this approach is that the same molecules are

used both for identification and validation of various targets. Most favourable, the binding molecules could also directly be used as therapeutic agents.

5 In another preferred embodiment, the template-displaying molecules are used to pull out the target molecules. This can for instance be achieved by selection against a cDNA library expressed on bacteriophage (libraries vs. libraries). By mixing a template-displaying molecule library with a cDNA li-
10 brary it will be possible to find binding pairs between the small molecules in the template-displaying molecule library and proteins from the cDNA library. One possibility is to mix a phage display library with a template display library and do a selection for either the phage or template library. The selected li-
15 brary is then plated to localized phage clones and the DNA coding for the phage and template displayed molecules can then be identified using PCR. Other types of libraries than cDNA could also be used such as nucleic acids, carbohydrates, synthetic polymer.

In another embodiment of the invention the template-displaying molecule technology can be used to account for in vivo and in vitro drug metabolism. That could include both phase I (activation) and phase II (detoxification) reac-
20 tions. The major classes of reactions are oxidation, reduction, and hydrolysis. Other enzymes catalyze conjugations. These enzymes could be used as targets in a selection process to eliminate displayed molecule that are prone to coordinate to these enzymes. The templates corresponding to these dis-
25 played molecules could subsequently be used to compete or eliminate these molecules when making template-displaying molecule libraries. These ob-
tained libraries will then be free of molecules that will have a tendency of binding to enzymes involved in phase I-II and possible be faster eliminated. For instance, a selection on each separate enzyme or any combination of
30 cytochrome P450 enzymes, flavin monooxygenase, monoamine oxidase, esterases, amidases, hydrolases, reductases, dehydrogenases, oxidases UDP-glucuronosyltransferases, glutathione S-transferases as well as other relevant enzymes could be performed to identify these binding molecules that

are prone to coordinate to these metabolic enzymes. Inhibitors are easily selected for due to their binding affinity but substrates need at least micro molar affinity to be identified.

5 Another interesting embodiment of this invention is the possibility to directly select for molecules that passively or actively becomes transported across epithelial plasma membrane, or other membranes. One possible selection assay is to use CaCO-2 cells, a human colon epithelial cell line, which is
10 testinal guts. The CaCO-2 assay involves growing a human colon epithelial cell line on tissue culture well inserts, such that the resultant monolayer forms a biological barrier between apical and basolateral compartments. The template-displaying molecule libraries are placed either side of the cell monolayer and the molecules that can permeate the cell monolayer is collected and amplified. This process can be repeated until active molecules
15 have been identified. Other cell line or setup of this assay is possible and is obvious for skill in the art.

A still further aspect of the present invention relates methods of selecting for
20 stability of the selected molecules. This could be performed by subjecting an enriched pool of binding molecules to an environment that will possibly degrade or change the structure of the binding molecules. Various conditions could be certain proteases or a mixture of protease, cell extract, and various fluids from for example the gastrointestinal gut. Other conditions could be
25 various salts or acid milieu or elevated temperature. Another possibility is to generate a library of known ligands and subject that library to stability tests and selection to identify stable molecules under certain conditions as describe above.

30 *Therapeutic applications*

The potential therapeutic applications of the invention are great. For example, the template-displaying molecule technology of the invention may be

used for blocking or stimulating various targets. A therapeutically relevant target is a substance that is known or suspected to be involved in a regulating process that is malfunctioning and thus leads to a disease state. Examples of such processes are receptor-ligand interaction, transcription-DNA interaction, and cell-cell interaction involving adhesion molecules, cofactor-enzyme interaction, and protein-protein interaction in intracellular signalling. Target molecule means any compound of interest for which a molecule ligand is desired. Thus, target can, for example, include a chemical compound, a mixture of chemical compounds, an array of spatially localized compounds, a biological macromolecule, such as DNA or mRNA, a bacteriophage peptide display library, a ribosome peptide display library, an extract made from biological materials such as bacteria, plants, fungi, or animal (*e.g.* mammalian) cells or tissue, protein, fusion protein, peptide, enzyme, receptor, receptor ligand, hormone, antigen, antibody, drug, dye, growth factor, lipid, substrate, toxin, virus, or the like etc., without limitation. Other examples of targets include, *e.g.* a whole cell, a whole tissue, a mixture of related or unrelated proteins, a mixture of viruses or bacterial strains or the like. etc., without limitation.

Therapeutic drug targets can be divided into different classes according to function; receptors, enzymes, hormones, transcription factors, ion channels, nuclear receptors, DNA, (Drews, J. (2000) Science 287:1960-1964). Among those, receptors, nuclear receptors, and metabolic enzymes constitute overwhelmingly the majority of known targets for existing drugs. Especially, G Protein-Coupled Receptors (GPCR) constitutes one of the most important classes of drug targets together with proteases for pharmacological intervention. Although the above examples are focused on the most relevant targets, it will be self-evident for a person skilled in the art that any other therapeutic target may be of interest.

The present invention employing the template-displaying molecule technology can be utilized to identify agonists or antagonists for all these classes of

drug targets, dependent on the specific properties each target holds. Most of the targets are possible to obtain in a purified form for direct selection procedures. Other targets have to be used when they are in their native environments such as imbedded cell surface receptors. In those situations the selection using the template-displaying molecule libraries can be performed using subtraction-selection described previously.

One specific application of the template-displaying molecule technology of the invention is to generate molecules that can function as antagonists, where the molecules block the interaction between a receptor and one or more ligands. Another application includes cell targeting. For example, the generated molecules recognizing specific surface proteins or receptors will be able to bind to certain cell types. Such molecules may in addition carry another therapeutic agent to increase the potency and reduce the side-effects (for example cancer treatment). Applications involving antiviral agents are also included. For example, a generated molecule, which binds strongly to epitopes on the virus particle, may be useful as an antiviral agent. Another specific application of the template-displaying molecule technology of the invention is to generate molecules that can function as agonists, where the molecules stimulate or activate a receptor to initiate a cellular signalling pathway.

Brief description of the figures

Fig. 1 shows the general principle for one embodiment of the present invention for the multi-step synthesis of templated molecules.

Fig. 2 shows the general structure of templates useful in the generation of a library.

Fig. 3 shows an example of design of templates for the generation of a library.

Fig. 4 shows examples of building blocks for use in the preparation of a library of templated molecules.

Fig. 5 shows further examples of building blocks.

Fig. 6 shows examples of the preparation of building blocks.

Fig. 7 shows examples of the preparation of building blocks starting from a 5'-NH₂ derivatized oligonucleotide.

5 Fig. 8 shows a general procedure of performing one embodiment for the formation of the templated molecule.

Fig. 8, example 1, shows an example of the embodiment shown in Fig. 8 involving light induced reaction between symmetrical building blocks.

Fig. 9 shows a general procedure of performing one embodiment for the formation of the templated molecule.

10 Fig. 10 shows a general procedure of performing one embodiment of the invention for the formation of a mixed polymer templated molecule.

Fig. 10, example 1, shows examples of simultaneous reaction and cleavage of neighbouring functional entities for the formation of (A) and alpha-peptide and (C) a polyamine..

15 Fig. 10, example 2, shows examples of simultaneous reaction and cleavage of neighbouring functional entities for the formation of (A) a peptoid, or an α - or β -peptide, and (B) a hydrazino peptide.

Fig. 11 depicts a templated synthesis of a polymer, using non-simultaneous reaction and cleavage.

20 Fig. 12 depicts formation of a templated molecule due to activation of reactive groups and partly release of the templated molecule for the template by ring-opening.

Fig. 13 shows the connection of two functional entities by the fill-in of connecting moiety.

25 Fig. 13, example 1, discloses an exemplification of Fig. 13, in which an imine is formed by a fill-in reaction.

Fig. 13, example 2, shows an exemplification of Fig. 13, in which an amide is formed.

30 Fig. 13, example 3, shows an exemplification of Fig. 13, in which an urea bonding is formed.

Fig. 13, example 3.1, shows an exemplification of Fig. 13 in which functional entities 13.3.1.A and 13.3.1.B are synthesised.

Fig. 13, example 4, shows the formation of chiral and achiral templated molecules.

Fig. 13, example 5, shows the formation of a phosphodiester bond by symmetric fill-in.

5 Fig. 13, example 6, shows the formation of a phosphodiester bond by a fill-in reaction, wherein the building block comprises a single reactive group.

Fig. 13, example 7, shows a pericyclic fill-in reaction.

Fig. 13, example 7.1, shows an exemplification of Fig. 13 in which functional entities 13.7.1.A and 13.7.1.B are synthesised.

10 Fig 14 shows a schematic representation of a fill-in reaction using asymmetric monomers.

Fig. 14, example 1, shows an asymmetric fill-in reaction using modified Staudinger ligation and ketone-hydrazide reaction.

15 Fig. 15 shows a schematic representation of a templated synthesis of a non-linear molecule.

Fig. 16 shows a representation of the templated synthesis of a non-linear molecule employing reactive groups of different classes and non-simultaneous reaction and cleavage.

Fig. 17 depicts a templated synthesis of a non-linear molecule, by exploiting the increased proximity effect that arises from a "migrating" scaffold.

Fig. 18 shows examples of the templated synthesis of non-linear molecules.

Fig. 19 shows a schematic representation of a templated synthesis, wherein the reaction step may be performed under conditions where specific annealing of building blocks to the template is inefficient.

Fig. 20 shows examples of various reactions types allowing simultaneous reaction and cleavage.

Fig. 21 shows examples of pairs of reactive groups (X) and (Y), and the resulting bond (XY).

Fig. 22 shows a schematic representation (panel A) of the zipper box principle and an example (panel B) of two building block.

Fig. 23 shows a schematic representation of various methods for increasing the proximity of functional entities of different building blocks.

Fig. 24 shows examples of the chemical constitution of a linker to be able to be cleaved.

Fig. 25 schematically shows the templated synthesis by generating a new reactive group.

Fig. 25, example 1, shows a method in which reactive groups generated in a first round subsequently are reacted with introduced reactive groups.

Fig. 26 shows examples of post-templating modifications of the templated molecule.

Detailed description of the invention

The following symbols are used in the figures to indicate general characteristics of the system: In figures 8; 10; 10, examples 1 and 2; 12; 12, examples 1; 13; 13, examples 1-7; 14; 14, example 1; 18; 20; 22; 23; 25; 25, example 1, a long horizontal line symbolizes a template. Coding region 1 symbolizes sequences that anneal to type 1 building blocks. Building blocks are symbolized as shown in figures 4-7. X/Y, S/T and P/Q represent pairs of reactive groups (where the reactive groups of one pair (e.g. X and Y) are partly or fully orthogonal to the reactive groups of other pairs (e.g. S/T, P/Q)). R_1, R_2, \dots, R_x symbolize functional groups. L and L_1, L_2, L_3, \dots represent cleavable linkers, where linkers of one group (e.g., L_1 -linkers) are cleavable under conditions where linkers of other groups (L_2, L_3, \dots) are not cleaved, or are cleaved less efficiently. The proximity effect that results from incorporating two building blocks on the same template, or alternatively, as a result of incorporating a building block on a template to which is attached a reactive group, may be enhanced by any of the methods described above or below that increases this effect. For example, in order to increase the efficiency and specificity of templated synthesis, the proximity effect may be increased by the introduction of zipper boxes in most of the general concepts described here.

In all the examples, the templated molecule may be coupled to the template through the non-covalent interaction of a monomer building block with the

template, or alternatively, through covalent or non-covalent coupling to the template, and may be located at either of the ends of the template, or anywhere on the template. The coupling reaction to the template may be performed before, during or after the synthesis of the templated molecule. For clarity, in some of the figures only the reaction step, not the cleavage step, has been included.

The figures included have been drawn so as to highlight specific set-ups. Obviously, any combination of the methods may be employed, in order to make linear, as well as non-linear molecules, to use reactive groups that lead to simultaneous cleavage, as well as reactive groups that do not lead to simultaneous cleavage, to use cleavable and non-cleavable linkers etc.

The protocol for an embodiment of a multi-step templated synthesis is shown in Fig. 1 and involves a number of steps that each result in the addition of one or more molecular moieties to a growing molecule that eventually becomes the templated molecule. Each of these steps can be divided into sub-steps. Initially, a number of templates (also called a library of templates) are provided. Each of the templates comprises a plurality of unique codons and a reactive group. Also, a plurality of different building blocks are provided, each of the building blocks comprises a functional entity separated from an anti-codon with a suitable linker. The anti-codon of a specific building block complements a unique codon of a template and is, therefore, capable under proper hybridisation conditions to anneal to the unique codon. The incorporation of building blocks is initiated by contacting the plurality of different templates with a subset of the entire amount of building blocks. The subset carries anti-codons which hybridise to unique codons of a distinct coding region. A connection between the reactive group of the template and the functional entity of the building blocks is obtained. In Fig. 1 the reactive group of the template is part of a building block (building block 1) and the said building block is hybridised to the template. In a preferred embodiment the building block 1 comprising the reactive group of the template and the second build-

ing block are contacted with the template simultaneously to allow for an efficient connection between the functional entities. The line between FE_1 and FE_2 symbolise a direct connection between the functional entities or an indirect connection via a bridging molecule entity. The molecule part formed by a connection of FE_1 to FE_2 is a nascent templated molecule, which may be added further functional entities resulting in a growing nascent templated molecule.

The propagation part of the method starts with the incorporation of a further building block (building block 3). The incorporation involves the hybridisation of a subset of the building blocks to the plurality of templates bearing the nascent templated molecule. The subset of building blocks is selected to have anti-codons which complement unique codons of the templates, said unique codons being in the vicinity of, preferably neighbouring to, unique codons hybridised to the building block(s) bearing the templated molecule. The functional entity of the further building block is able to form a chemical connection to the nascent templated molecule through the reaction of a reactive group attached to the functional entity. The linkage between one or more of the functional entities and the corresponding anti-codons may be cleaved if desired and the incorporation of a new building block may be performed. In the example illustrated in Fig. 1 only three functional entities are connected in the templated molecule. However the propagation step may be conducted as many times as appropriate to obtain the desired templated compound.

As a terminal phase the linkers connecting functional entities/templated molecule and anti-codons may be cleaved. The complex comprising the templated molecules (specific compositions or sequences of molecular moieties, the identity of which is determined by the template) attached to the templates that templated their synthesis, can now be taken through a screening process. This process leads to an enrichment of templated molecules complexes with appropriate characteristics. The isolated complexes may now be enriched by amplification of the templates, and a new round of templated

synthesis and screening can be performed. Eventually, the templated molecules may be identified by characterization of the corresponding templates.

The stages of the process involving incorporation of building blocks may be mediated by chemicals, or enzymes such as polymerase or ligase. For example, the anti-codon part of the building blocks may be nucleotide-derivatives that are incorporated by a polymerase. Incorporation may also be solely by hybridization of building blocks to the template. If the template is a DNA molecule, the template may comprise primer binding sites at one or both ends (allowing the amplification of the template by e.g. PCR). The remaining portion of the templates may be of random or partly random sequence.

The reaction stage of the method involves reactions between the incorporated building blocks, thereby forming chemical connections between the functional entities. The chemical connection can be a direct chemical bond or the connection can be established through a suitable bridging molecule.

The optional cleavage step involves cleaving some, all but one, or all of the linkers that connect the functional entities and anti-codons. In Fig. 1 the templated molecule is displayed by cleaving the linkers of the second and third functional entities, while maintaining the linker from the first building block.

Subsequent to the production of library according to the invention a selection is performed. The selection or screening involves enriching the population of template-templated molecule pairs for a desired property. For example, passing a library of templated molecule-template complexes over a solid phase to which a protein target has been immobilized, and washing unbound complexes off, will enrich for complexes that are able to bind to the protein.

The selection may be performed more than once, for example with increasing stringency. Between each selection it is in general preferable to perform an amplification. The amplification involves producing more of the template-templated molecule complexes, by amplification of the template or complementing template, and producing more of the template-templated molecule pairs, for further rounds of selection/screening, or for sequencing or other characterization. For example, if the template is a DNA strand, the template may be amplified by PCR, where after the templated synthesis can be performed using the amplified DNA, as described above.

Cloning and sequencing may also be useful techniques and involve the cloning of the isolated templates or complementing templates, followed by characterization. In some cases, it may be desirable to sequence the population of isolated templates or complementing templates, wherefore cloning of individual sequences is not required.

In Fig. 2, in the upper part of the figure, the general structure of a template is shown. The templates comprise x coding regions. Each coding region has a unique sub-structure which differentiates it from some or all of the other coding regions. Shown below the general structure of a template are specific templates. A given specific template carries a specific set of x unique codons. A unique codon specifies (by way of interaction with a specific anti-codon of a building block) a specific functional entity. The unique codons 1.1, 1.2, 1.3,, 1.m are all examples of coding region 1 sequences. The general design of the templates therefore enables the templated incorporation of building blocks, in the sense that a sub-set of building blocks can be added that will only be incorporated at the same position on the template (i.e., coding region 1 if the building blocks have anti-codons that are complementary to the unique codons of codon region 1).

Fig. 3 Shows an example of a design of templates and anti-codons for oligonucleotide-based building blocks. Section A discloses the general

structure of a set of templates carrying 6 coding regions, each containing a partly random sequence (X specifies either C or G), and a constant sequence that is identical for all sequences in the group (e.g., all coding region 1 sequences carries a central ATATT sequence). By using C and G only (or, alternatively, A and T only), the building blocks that are complementary to coding regions 1 have very similar annealing temperatures wherefore mis-annealing is insignificant. The attachment point of the linker that connects the anti-codon and the functional entity is not specified in the figure. Ideally, the linker is attached to the constant region of the anti-codon, in order to avoid bias in the annealing process.

Section B of Fig. 3 shows examples of codon and anti-codon sequences. Example codon 1 and codon 6 sequences are shown. The example codon 1 sequence represents one specific sequence out of 1024 different sequences that anneal specifically to the complementary anti-codon 1 sequences; the example codon 6 sequence represents one specific sequence out of 128 different sequences that anneal to the complementary anti-codon 6 sequences.

Fig. 4 illustrates different general designs of building blocks. A building block comprises or essentially consists of a functional entity, connected through a (cleavable) linker to an anti-codon. Panel A shows a building block with one reactive group (X), connecting the functional group (R_x) with the anti-codon. This type of building block may be used for the simultaneous reaction and cleavage protocol (e.g. figures 9 and 15). The functional entity in this example comprises one reactive group, and a functional group R_x , also called a functionality. The reactive groups typically become part of the templated molecule. Panel B shows a building block with two reactive groups (X and Y), connecting the anti-codon and the functional group (R_x). The functional entity in this example comprises two reactive groups that are both part of the moiety that links the anti-codon and functional group, R_x . Panel C shows a building block with a reactive group (X) connecting R_x and the anti-codon, and a

reactive group (Y) attached to the R_x group. This type of building block may be used in the simultaneous reaction and cleavage protocol (e.g., Fig. 9 and 10). The functional entity comprises two reactive groups X and Y, where X is part of the linker, and Y is attached to the functional group R_x . Panel D shows a building block with one reactive group (X). The reactive group (X) does not link the functional group (R_x) and the complementing element. A cleavable linker (L) is provided in order to release the functional entity from the anti-codon. This type of building block may be used in protocols that require cleavage of the linker after the reactive groups of the functional entities have reacted (e.g., Fig. 12). Panel E disclose a building block with four reactive groups and a functional group R_x . The four reactive groups and the functional group R_x may serve as a scaffold, onto which substituents (encoded by building blocks bound to codons on the same template) are coupled through reaction of reactive groups (X) of other building blocks with the reactive groups (Y) (e.g., figure 15). In this example, no cleavable linker is indicated. Therefore, after the templating reactions the templated molecule is attached to the template through the linker of this building block.

In Fig. 5 three different building blocks are depicted. Building block A comprises an anti-codon (horizontal line), which may be an oligonucleotide, to which a linker carrying the functional entity is attached to the central part. The portion of the linker marked "a" may represent a oligonucleotide sequence to which a single stranded nucleotide may be annealed in order to make the linker more rigid, or alternatively, "a" may represent a zipper box sequence of nucleotides or other type of zipper box moiety. The vertical line may represent a PEG (polyethylene glycol) linker, oligonucleotide linker, or any other linker that provides the functional entity with the appropriate freedom to interact productively with a functional entity of a building block annealed to the same template during the templating process. In building block, the linker is attached to the terminus of the anti-codon. The anti-codon and the linker may be one continuous strand of an oligonucleotide. The horizontal part here represents the anti-codon, and the vertical part

represents the linker. The linker may contain a moiety "a" that functions as a zipper box (see Fig. 22), a rigid linker, or an annealing site for another entity that rigidifies the linker upon annealing. In building block C of Fig. 5 the linker and anti-codon may be a continuous strand of an oligonucleotide. Attached to the linker is a nucleophile "Nu" which may react with a functional entity. This may be used as an anchorage point for the templated molecule. Building block C may preferably be used as the starting or the terminal building block. When used in the initial stage of the production of the complex comprising the templated molecule, building block C may provide the template with a reactive group to which the functional entities may be attached in the growing templated molecule. In a further embodiment of the invention "Nu" of building block C represents any reactive group able to participate in a reaction resulting in the formation of a connection to a functional entity of a building block.

Fig. 6 shows five different general methods for the preparation of building blocks. The general methods involves the coupling of the functional entities to oligonucleotide-based building blocks. Reactions and reagents are shown that may be used for the coupling of functional entities to modified oligonucleotides (modified with thiol, carboxylic acid, halide, or amine), without significant reaction with the unmodified part of the oligonucleotide. As an alternative approach, the functional entity may be synthesized as phosphoramidite precursor, which can then be used for oligonucleotide synthesis by standard methods, resulting in an oligonucleotide-derivative carrying a functional entity.

Fig. 7 shows the design and synthesis of exemplary building blocks. Panel A shows a general synthesis scheme for building blocks using DNA oligonucleotide as codon, and coupling amines and carboxylic esters. The oligonucleotide is purchased with an amine coupled to e.g. the base at a terminal position of the oligo. By addition of EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride) and NHS (N-

hydroxysuccinimide), the oligonucleotide is coupled to the building block through an amide bond. Panel B shows specific synthesis schemes for the generation of specific classes of building blocks.

Fig. 8 illustrates an embodiment for the templated synthesis of a polymer. X and Y are reactive groups of the functional entity. X and Y may be different kinds of reactive groups (e.g., amine and carboxylic acid), of the same kind but different (e.g., different primary amines or a primary amine and a secondary amine), or identical. Reaction of X with Y to form XY either happens spontaneously when the building block has been incorporated, or is induced by a change of conditions (e.g. pH), or by the addition of an inducing factor (chemical or UV exposure, for example)

5 Fig. 8, ex.1. shows light-induced reaction between symmetric coumarin-derivatives. Light-induced reaction of the coumarin units, followed by cleavage of the linker, result in a ring structure. Examples of functional groups (phosphate and carboxylic acid) are shown. The building blocks are said to be symmetric because the two reactive groups, two coumarin units, are of the same reactivity (in fact, in this example are identical).

Fig. 9 shows an embodiment for templated synthesis of a polymer. A population of templates, each carrying four codons are incubated with two sets of building blocks (carrying anti-codons 1 and 2, respectively), at a temperature that ensures efficient and specific annealing of anti-codons type 1 to coding regions 1, and efficient and specific annealing of anti-codons type 2 to coding regions 2. After annealing, the excess building blocks may optionally be removed. If desired, reactive groups may be deprotected (and thus activated for reaction) at this step. Then building block-template complexes are incubated under conditions that allow the reactive groups of the building blocks (i.e., reactive groups X and Y) to react. This leads to a transfer of the functional group R1 from building block 1 to building block 2, and thus results in the formation of a dimeric polymer carrying two functional

groups, R1 and R2. The process is then repeated, i.e. a third monomer (with anti-codon type 3) is added, and after annealing to coding region 3, excess building block is removed, and the reaction between X and Y now leads to the formation of a trimeric polymer, coupled to the building block annealed to coding region 3. Once more, the process is repeated with building blocks of type 4, resulting in the formation of a tetrameric polymer.

The reactive groups X and Y used in this scheme thus have two functions: i) reaction between X and Y leads to coupling of the corresponding functionalities (e.g., R1 and R2), and simultaneously, ii) the linker between R1 and the anti-codon is cleaved. Examples of reactive groups X and Y with such characteristics (i.e., the ability to simultaneously react and cleave) are shown in Fig. 20. By appropriate choice of X and Y, the nascent polymer is migrated down the template, from building block to building block, as it is being synthesized. For example, by choosing X = ester (COOR), and Y = amine (NH₂), the nucleophilic attack of the amine on the ester leads to transfer of the upstream functionality (e.g., R₁) to the downstream building block (e.g., carrying anti-codon type 2). This ensures the highest possible effect of proximity with this set-up (i.e., in each step, the reacting X and Y are carried on neighbouring monomers).

If desired, the templated polymer may be coupled to the template through the non-covalent interaction of a building block with the template (in the example given, through the interaction of building block 4 with the template), or alternatively, through covalent coupling to a reactive group on the template, located at either of the ends of the template, or anywhere on the template sequence. In the latter case, the coupling reaction to the template may be performed before, during or after the synthesis of the polymer.

Fig. 10 shows the templated synthesis of a mixed polymer. The most noticeable difference, when compared to the embodiment shown in Fig. 9 is that the reactive groups on the individual building blocks are different. The

pairs of reactive groups (X/Y, S/T, and P/Q) are chosen so that the reaction of X and Y, S and T, P and Q, respectively, results in transfer of a functional group from one building block to another (i.e., the reaction both mediates the coupling of the two functional groups and the cleavage of the linker that initially connects one of the functional groups to the anti-codon). Example pairs of reactive groups that mediate this simultaneous reaction and cleavage are shown in Fig. 20.

Fig. 10, example 1 shows two methods of obtaining different classes of compounds using simultaneous reaction and cleavage. In panel A, the formation of an alpha-peptide is disclosed and in panel C the synthesis of a polyamine is shown.

In panel A, two building blocks are incorporated by hybridization to the template. One of the building blocks is an oligonucleotide to which has been appended a thioester. The other building block is an oligonucleotide to which has been appended an amino acid thioester. The amine of the latter building block attacks the carbonyl of the other building block. This results in formation of an amide bond, which extends the peptide one unit. When the next amino acid thioester building block is incorporated, this may attack the thioester carbonyl, resulting in cleavage of the dipeptide from the anti-codon, to form a tripeptide. This process is repeated until the desired peptide has been generated. Importantly, as the reaction in each step is between the incoming subunit-precursor and the subunit of the nascent polymer that is closest to the linker that connects it to the anti-codon, the geometry of the nucleophilic attack remains unchanged. The reactivity of the amine with the ester may be tuned in several ways. Parameters that will affect the reactivity include: (i) pH and temperature, (ii) nature of ester (thio-, phospho-, or hydroxy-ester); (iii) the nature of the substituent on the sulfur (see panel B below).

The general scheme presented here can be applied to most nucleophilic reactions, including formation of various types of peptides, amides, and amide-like polymers (e.g., mono-, di-, tri-, and tetra-substituted α -, β -, γ -, and Ω -peptides, polyesters, polycarbonate, polycarbamate, polyurea), using similar functional entities.

Panel B shows four different thioesters with different substituents on the sulphur and therefore different reactivity towards nucleophiles.

Panel C relates to the formation of a polyamine. Using the same principle as in (A), a polyamine is formed.

Fig. 10, example 2 shows simultaneous reaction and cleavage for two reactions. In reaction A a peptoid or an α - or β -peptide is formed, and in reaction B a hydrazino peptide is formed.

In reaction A, two building blocks are initially incorporated, one of which carries both a nucleophile (an amino group) and an electrophile (e.g. an ester); the other building block only carries an electrophile (e.g. a thioester). As a result, the nucleophilic amine will attack the electrophile of the building block attached to the same template. As a result, a dimeric structure is formed, linked to building block that initially carried the amine. Upon sequential addition of building blocks, the linear structure grows, and eventually the desired templated molecule (a peptoid or an α - or β -peptide) has been formed.

The reaction B follows the same line as in A, except that hydrazine-peptide precursor building blocks are used, leading to the formation of hydrazino peptides.

Fig. 11 shows a general reaction scheme for templated synthesis of a polymer, using non-simultaneous reaction and cleavage. In this scheme, the

reaction of the reactive groups (e.g., X and Y) does not in itself lead to cleavage, wherefore the functional entity is coupled to the anti-codon via a cleavable linker. Therefore, each addition of a subunit to the growing polymer involves two steps. First, the reactive groups X and Y react to form a bond XY. Then, in a separate step, a cleavable linker L is cleaved, which releases one of the functional entities from the anti-codon. By alternating between two types of cleavable linkers (cleavable under different conditions) one may achieve migration of the nascent polymer down the template, like described in fig. 9 and 10. This ensures the highest possible effect of proximity with this set-up (i.e., in each step, the reacting X and Y are carried on neighbouring monomers). In the example, some or all of the reactive pairs may be of the same kind (e.g., $X/Y = S/T = P/Q$).

Example reactions that do not mediate simultaneous reaction and cleavage are shown in fig. 21. Any combination of cleavable and non-cleavable linkers may be used, dependent on the nature of the reactive groups in the functional entities (e.g., dependent on whether the reaction involves a release from the anti-codon).

Fig. 12 relates to activation of reactive groups and release from anti-codon by ring-opening.

Reaction of the initiator with X in the ring structure opens the ring, resulting in activation of Y. Y can now react with X in a neighboring functional entity. As a result of ring-opening, the functional entities are released from the anti-codons. If the zipper-box principle is applied to this set-up (where each additional building block added reacts with the nascent templated molecule attached to the initiator), the initiator linker must carry half of the zipper (e.g., the "sense strand"), and all the building blocks must carry the other half of the zipper-box (the "anti-sense strand").

Fig. 12, example 1. Ring-opening of N-thiocarboxyanhydrides, to form β -peptides.

After incorporation of two building blocks, where one of the building blocks carry an initiator reactive group (or incorporation of one building block next to a covalently attached initiator molecule), the initiator is activated, for example by deprotection or by an increase in pH. The primary amine then attacks the carbonyl of the N-thiocarboxyanhydride (NTA) unit. As a result, CSO is released, and a primary amine is generated. When the next building block is incorporated, this amine will react with the NTA, and eventually when all the building blocks have been incorporated and the NTA units have reacted, a β -peptide will have formed. Finally, the linkers that connect the β -peptide to the anti-codons are cleaved, resulting in a β -peptide attached to its template through one linker.

A number of changes to this set-up can be envisaged. For example, instead of thiocarboxyanhydrides, one might use carboboxyanhydrides. The initiator might be protected with a base- or photolabile group. If a base-labile protection group is chosen, the stability of the carboxyanhydride must be considered. At higher pH it may be advantageous to use carboxyanhydrides rather than thiocarboxyanhydrides. Other types of peptides and peptide-like polymers (e.g., mono-, di-, tri-, and tetra-substituted α -, β -, γ -, and Ω -peptides, polyesters, polycarbonate, polycarbamate, polyurea) can be made, using a similar scheme. For example, α -peptides can be made by polymerization of 5-membered carboxyanhydride rings.

Fig. 13 shows the principle of symmetric fill-in (symmetric XX building blocks). The fill-in reaction occurs between the reactive groups ("X" in the figure) and bridging molecules "Y-Y" in figure).

For clarity, only the reaction (not the cleavage) is shown in the figure. X represents the reactive groups of the functional entity. In this case the two reactive groups are of the same kind. (Y-Y) is added to the mixture before, during or after incorporation of the building blocks.

Fig. 13, ex.1 shows imine formation by fill-in reaction.

Dialdehyde is added in excess to incorporated diamines. As a result, an imine is formed. In the example, the templated molecule carries the following functional groups: cyclopentadienyl and hydroxyl.

Fig. 13, example 2 shows an example of amide formation using symmetric fill-in. After incorporation of two building blocks each carrying a di-amine, non-incorporated building blocks may be removed. Then EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide), and dicarboxylic acid is added in excess to the primary amines of the building blocks. Alternatively, a di-(N-hydroxy-succinimide ester) may be added in excess. At a pH of 7-10, this will lead to the formation of two amide bonds linking the functional entities. After reaction, excess reagents may be removed by dialysis, precipitation of the building blocks and template, gel filtration or by other means that separate the reagents from the building blocks. When the process of incorporation-and-reaction has been repeated a number of times, and the desired molecule has been templated, the linkers (L) may be cleaved, and, if functional groups have been masked by protection groups (PG), these functional groups can be deprotected to expose the functional groups. Appropriate protecting groups would be for example Boc-, Fmoc, benzyloxycarbonyl (Z, cbz), trifluoroacetyl, phthaloyl, or other amino protecting groups described e.g. in (T. W. Green and Peter G. M. Wuts (1991), *Protective Groups in Organic Synthesis*).

An alternative route to amide-bonded functional entities would be to incorporate building blocks carrying di-carboxylic acids, and then add diamines, NHS and EDC. Alternatively, the building blocks could carry N-hydroxy-succinimidyl (NHS) esters, which would react with the added amines without the need to add EDC and NHS.

Fig. 13, example 3 shows an example of urea-bond formation. The functional entities of the incorporated building blocks react with phosgen or a phosgen-equivalent such as CDI to form a polyurea. Formaldehyde may

also be used. The linkers are cleaved and the protected hydroxyl is deprotected. Appropriate leaving groups (Lv) are chloride, imidazole, nitrotriazole, or other good leaving groups.

Fig. 13, example 4 shows the formation of chiral and achiral templated molecules. In this example, the functional group Rx is used as a cleavable linker, that generates the desired functional group upon activation. In both reaction A and reaction B, a urea-bond is formed.

In reaction A, the functional group is attached to the backbone via a chiral carbon. The hydrogen on this carbon is drawn to emphasize this. Before bond formation, there is free rotation about the bond connecting the chiral carbon and the functional group. When the reactive groups (the amines) react with the phosgen equivalent (e.g., a carbonyldiimidazole) to form the templated molecule, the building blocks may be inserted in either of two orientations (as indicated by the position of the hydrogen, left or right). As a result, each encoded residue of the templated molecule will have two possible chiral forms. If the templated molecule was e.g. a pentameric polyurea (formed from five functional entities), this molecule would have $2^5 = 32$ stereoisomers. In certain cases it may be advantageous to incorporate such additional structural diversity in the library (for example when a low building block diversity is employed). In other cases such additional diversity is not desirable, as the screening efficiency may become compromised, or it may become too difficult to determine the identity of a templated molecule that has been isolated in a screening process.

In reaction B, the chiral carbon of reaction A has been replaced by a nitrogen. As a result, the resulting templated molecule is achiral, i.e. the template encodes one specific structure.

Fig. 13, example 5 shows the formation of a phosphodiester bond by the principle of symmetric fill-in. The incorporated building blocks react with the

activated fill-in molecule to form a phosphodiester bond. Then the linkers are cleaved, releasing the templated molecule from its template. An example of an appropriate leaving group (Lv) is imidazole.

Fig. 13, example 6 shows phosphodiester formation with one reactive group in each building block.

Upon addition of a dihydroxylated compound such as 1,3-dihydroxypyridine, a phosphodiester bond is formed. Finally, the functional group Rx is liberated from the anti-codon by cleavage of the protection groups/cleavable linker that connected it to the anti-codon.

Fig. 13, example 7 shows the an example of a pericyclic fill-in reaction. First, two building blocks are incorporated. Then 1,4-benzoquinone is added in excess, resulting in the formation of a polycyclic compound. A third building block is added, reacted with the 1,4-benzoquinone, and this process is repeated a number of times until the desired templated molecule has been generated. Finally, all but one of the linkers that connect the templated molecule to the anti-codon, are cleaved.

Fig. 14 relates to asymmetric fill-in using XS building blocks.

A fill-in reaction between reactive groups (X and S) and bridging molecules (T-Y) is shown. For clarity, only the reaction (not linker cleavage) is shown. X and S represent the reactive groups of the functional entity. In this case the two reactive groups are not of the same kind. (T-Y) is added to the mixture before, during or after incorporation of the building blocks. Likewise, significant reaction between X and Y, and between S and T may take place during or after incorporation of the building blocks.

Fig. 14, example 1 shows an example of asymmetric fill-in by modified Staudinger ligation and ketone-hydrazide reaction. The reactive groups X and S of the building blocks are azide and hydrazide. The added molecule that fills the gaps between the building blocks carry a ketone and a

phosphine moiety. The reactions between a ketone and a hydrazide, and between an azide and a phosphine, are very chemoselective. Therefore, most functional groups Rx can be employed without the need for protection during the reactions. Examples for the molecular moieties R, R₁, X and Y may be found in (Mahal et al. (1997), Science 276, pp. 1125-1128; Saxon et al. (2000), Organic Letters 2, pp. 2141-2143).

Fig. 15 shows a general reaction scheme for templated synthesis of a non-linear molecule. A template carrying four codons is mixed with two building blocks. The functional entity of one building block comprises a reactive group X and a functional group R₁. The other building block comprises three reactive groups Y and a functional group R₂. The building block bound to codon 2 is here called the scaffold, as the functional groups are transferred to this building block during the templating process.

After incubation at a temperature that ensures efficient and specific annealing of the two building blocks to their respective codon, and optionally, excess building block has been removed, X is brought to react with one of the reactive groups Y, for example by changing the conditions, by deprotecting X or Y, or by simply exploiting the pronounced proximity of X and Y groups when the building blocks are bound to the template.

In this scheme, X and Y have been chosen so as to allow simultaneous reaction and cleavage. Thus, as a result of the reaction between X and Y, the substituent group (functional group) R₁ is transferred to the scaffold. Example reactive groups X and Y that mediate simultaneous reaction and cleavage are given in figure 20. Any pair of reactive groups X and Y that mediates simultaneous reaction and cleavage can be used in this scheme, i.e., different X/Y pairs may be used at each substituent position.

Annealing and reacting of two more building blocks lead to the formation of a scaffolded molecule carrying three substituents (R₁, R₃ and R₄). The identity

of the substituents is determined by the codons of the template to which the scaffolded molecule is attached.

Figure 16 shows templated synthesis of a non-linear molecule, employing reactive groups of different kinds, and non-simultaneous reaction and cleavage. The reactive groups X, S, P and Y, T, Q may be of different kinds, and the bonds formed (XY, ST, and PQ) therefore may be of different kinds.

After reaction and then cleavage of the linker L (that attaches the functional entity of the first building block to the anti-codon), the substituent (functional group) R1 is transferred to the second building block (the scaffold). Thus, relative to the synthesis scheme of figure 15, here an additional step of linker cleavage is required. After repeating the processes of annealing, reacting and cleavage a number of times, a scaffolded molecule has been formed carrying encoded substituents. The identity of the substituents is determined by the codons of the template to which the scaffolded molecule is attached. The position of the substituents are determined by the identity of the reactive groups Y, T and Q of the scaffold, and therefore indirectly determined by the identity of the codon to which the scaffold building block anneals. Therefore, in this set-up, the identity and position of the substituents, as well as the identity of the scaffold, is determined by the sequence of the template. The reactive pairs may also be of the same kind (e.g., $XY = ST = PQ$).

Fig. 17 discloses the principle of templated synthesis of a non-linear molecule, by exploiting the increased proximity effect that arises from a "migrating" scaffold. In this set-up, the templated molecule migrates down the template as it is being synthesized. This is made possible by the use two different linkers L_x and L_y , cleavable under different conditions. As a result, a high proximity is maintained throughout the templating process, as the building blocks that react in each reaction step are bound to adjacent coding regions on the template.

Fig. 18 shows the templated synthesis of various non-linear molecules.

- (A) Three building blocks are added and reacted one at a time. Each building block comprises an activated ester (reactive group, (X)) where the ester moiety carries a functional group Rx. Upon reaction between the esters and the amines on the scaffold (scaffold is covalently attached to the template), amide bonds are formed, and the Rx groups are now coupled to the scaffold via amide bonds. This is thus an example of simultaneous reaction (amide formation) and cleavage (release of the Rx moiety from the anti-codon), see e.g. fig.15.
- (B) Analogously to (A), three amines react with three esters to form three amide bonds, thereby coupling the functional groups Rx to the scaffold moiety. However, as opposed to (A), the scaffold is here encoded by the template, and therefore the scaffold is here part of the functional entity of a building block.
- (C) Three building blocks are used. The nucleophilic amine (covalently attached to the template) attacks the ester carbonyl of the building block bound to coding region 3; the amine of the third monomer attacks the thioester of the next incorporated building block, and after incorporation of the third building block, the Horner-Wittig Emmans reagent of the building block reacts with the aldehyde of the third monomer under alkaline conditions. This forms the templated molecule. The double bond may be post-templating modified by hydrogenation to form a saturated bond, or alternatively, submitted to a Michael addition.
- (D) The thiol of the scaffold reacts with the pyridine-disulfide of the incorporated building block. The amine of the scaffold reacts with the ester of the second incorporated building block. The double nitrile-activated alpha-position is acylated by the thioester of the next

building block in the presence of base. Finally, the aryl iodide undergoes Suzuki coupling with the aryl boronate of monomer 4 to yield the biaryl moiety.

(E) The incorporated building block acylates one of the primary amines. The aryl iodide undergoes a Suzuki coupling by reaction with the next building block, and the benzylic amine is acylated by last incorporated building block.

(F) Acylation of the hydrazine followed by cyclization leads to formation of a hydroxypyrazole. After incorporation of the second building block, the aryl bromide undergoes Suzuki coupling with the aryl boronate. Finally, the aldehyde reacts with the Horner-Wittig-Emmons reagent of the building block that is next incorporated, to yield an alpha, beta-unsaturated amide, which may be further modified or functionalized by either reduction with $H_2/Pd-C$ or Michael addition with nucleophiles. Alternatively, a fourth building block might be used to template the coupling of a nucleophilic substituent at the double bond position.

Fig. 19 shows a general procedure of templated synthesis, wherein the reaction step may be performed under conditions where specific annealing of building blocks to the template is inefficient.

It may be desirable to perform the reaction step (or one of the other steps) under conditions where annealing of building blocks is inefficient. To solve this potential problem, one may covalently link the incorporated building blocks, either chemically or by using a ligase (when the anti-codon comprises an oligonucleotide) or a polymerase (when the anti-codon is e.g. a nucleotide). In this set-up, the template is designed to fold back on itself.

In step 1, the two incorporated building blocks are incorporated and may be ligated together, and be linked to the template, during or after their incorporation. If desired, the conditions may now be changed to increase the efficiency of the reaction step that follows. Then, in step 2, the reactive groups X and Y are brought to react. Because the building blocks are covalently attached to each other (and to the template), the reaction can be performed under conditions where annealing of the building blocks to the template is inefficient. Reaction conditions that may not be compatible with efficient annealing and double helix structure include organic solvents, low salt and high temperature, all of which may be used with the set-up described in this figure.

After step 2 (reaction), the conditions are changed again, in order to allow efficient incorporation and covalent linkage of the next building block (step 3). This cycling between conditions that allow incorporation and ligation, and that allow reaction, is continued until the desired number of building blocks have been incorporated and reacted. Finally, some of the linkers are cleaved to give the templated molecule. As described above, the covalent coupling of the building blocks to each other allows the reaction between their reactive groups to be performed under more diverse conditions than would otherwise be possible. In addition, covalent coupling between building blocks makes it possible to use anti-codons comprising shorter recognition sequences. When the anti-codon comprises an oligonucleotide, it is generally preferred to use an oligonucleotide of at least fifteen nucleotides during incorporation, in order to obtain high efficiency of incorporation. However, if a ligase or chemical is used to covalently couple the building blocks, a shorter oligonucleotide (4-8 nucleotides) may be used. This will bring the reactive groups X and Y into closer proximity, and increase the local concentration of reactive groups dramatically: If the distance between the reactive groups is decreased from 16 nucleotides to 4 nucleotides, this will increase the local concentration $4^3 = 64$. Everything else being equal, this will increase the rate of the reaction by 64-fold.

In order to change between conditions that allow incorporation and covalent coupling between building blocks, and conditions that allow the reaction to occur efficiently, the templates may be attached to a solid phase material (e.g., streptavidin beads if the templates are biotinylated), or the templates (with the building blocks associated to them) may be precipitated and resuspended in appropriate buffer during the steps of incorporation and reaction.

Fig. 20 shows various reaction types allowing simultaneous reaction and activation. Different classes of reactions are shown which mediate translocation of a functional group from one monomer building block to another, or to an anchorage point. The reactions have been grouped into three different classes: Nucleophilic substitutions, addition-elimination reactions, and transition metal catalyzed reactions. These reactions are compatible with simultaneous reaction and activation.

- (A) Reaction of nucleophiles with carbonyls. As a result of the nucleophilic substitution, the functional group R is translocated to the monomer building block initially carrying the nucleophile.
- (B) Nucleophilic attack by the amine on the thioester leads to formation of an amide bond, in effect translocating the functional group R of the thioester to the other monomer building block.
- (C) Reaction between hydrazine and β -ketoester leads to formation of pyrazolone, in effect translocating the R and R' functional groups to the other monomer building block.
- (D) Reaction of hydroxylamine with β -ketoester leads to formation of the isoxazolone, thereby translocating the R and R' groups to the other monomer building block.
- (E) Reaction of thiourea with β -ketoester leads to formation of the pyrimidine, thereby translocating the R and R' groups to the other monomer building block.

- (F) Reaction of urea with malonate leads to formation of pyrimidine, thereby translocating the R group to the other monomer building block.
- (G) Depending on whether $Z = O$ or $Z = NH$, a Heck reaction followed by a nucleophilic substitution leads to formation of coumarin or quinolinon, thereby translocating the R and R' groups to the other monomer building block.
- (H) Reaction of hydrazine and phthalimides leads to formation of phthalhydrazide, thereby translocating the R and R' groups to the other monomer building block.
- (I) Reaction of amino acid esters leads to formation of diketopiperazine, thereby translocating the R group to the other monomer building block.
- (J) Reaction of urea with α -substituted esters leads to formation of hydantoin, and translocation of the R and R' groups to the other monomer building block.
- (K) Alkylation may be achieved by reaction of various nucleophiles with sulfonates. This translocates the functional groups R and R' to the other monomer building block.
- (L) Reaction of a di-activated alkene containing an electron withdrawing and a leaving group, whereby the alkene is translocated to the nucleophile.
- (M) Reaction of disulfide with mercaptane leads to formation of a disulfide, thereby translocating the R' group to the other monomer building block.
- (N) Reaction of amino acid esters and amino ketones leads to formation of benzodiazepinone, thereby translocating the R group to the other monomer building block.
- (O) Reaction of phosphonates with aldehydes or ketones leads to formation of substituted alkenes, thereby translocating the R'' group to the other monomer building block.

- (P) Reaction of boronates with aryls or heteroaryls results in transfer of an aryl group to the other monomer building block (to form a biaryl).
- (Q) Reaction of arylsulfonates with boronates leads to transfer of the aryl group.
- (R) Reaction of boronates with vinyls (or alkynes) results in transfer of an aryl group to the other monomer building block to form a vinylarene (or alkynylarene).
- (S) Reaction between aliphatic boronates and arylhalides, whereby the alkyl group is translocated to yield an alkylarene.
- (T) Transition metal catalysed alpha-alkylation through reaction between an enolether and an arylhalide, thereby translocating the aliphatic part.
- (U) Condensations between e.g. enamines or enolethers with aldehydes leading to formation of alpha-hydroxy carbonyls or alpha,beta-unsaturated carbonyls. The reaction translocates the nucleophilic part.
- (V) Alkylation of alkylhalides by e.g. enamines or enolethers. The reaction translocates the nucleophilic part.
- (W) [2+4] cycloadditions, translocating the diene-part.
- (X) [2+4] cycloadditions, translocating the ene-part.
- (Y) [3+2] cycloadditions between azides and alkenes, leading to triazoles by translocation of the ene-part.
- (Z) [3+2] cycloadditions between nitriloxides and alkenes, leading to isoxazoles by translocation of the ene-part.

Fig. 21 shows pairs of reactive groups (X) and (Y), and the resulting bond (XY).

A collection of reactive groups that may be used for templated synthesis as described herein are shown, along with the bonds formed upon their reaction. After reaction, cleavage may be required (e.g., see fig. 8).

Fig. 22 shows a method of increasing the proximity effect of the template: The Zipper-box.

Panel A discloses linkers carrying oligonucleotide zipper boxes (a) and (b) that are complementary. By operating at a temperature that allows transient interaction of (a) and (b), the reactive groups X and Y are brought into close proximity during multiple annealing and strand-melting events, which has the effect of keeping X and Y in close proximity in a larger fraction of the time than otherwise achievable. Alternatively, one may cycle the temperature between a low temperature (where the zipper boxes pairwise interact stably), and a higher temperature (where the zipper boxes are apart, but where the anti-codon remains stably attached to the codon of the template). By cycling between the high and low temperature several times, a given reactive group X is exposed to several reactive groups Y, and eventually will react to form an XY bond. As a final alternative, the temperature may be kept appropriately low that the two strands of the zipper-box (a and b) are stably associated. Independent on which of these protocols is followed, the building blocks must be added to the reaction mix at an appropriately high temperature where the interaction between the codon and anti-codon is specific. Once the building blocks have been specifically associated with the template, the temperature can be lowered, and the alternative protocols described above followed, in order to achieve a high reaction efficiency.

When the anti-codon is an oligonucleotide (e.g., DNA, RNA) or oligonucleotide analog (e.g., PNA, LNA), it may be practical to use a continuous nucleotide strand, comprising both the anti-codon, linker and zipper-box (see (B) below).

- 5 Panel B shows sequences of two DNA oligo-based building blocks. The anti-codon ("annealing region"), linker and zipper-box are indicated. Thus, in this example, one linear DNA molecule constitutes the anti-codon, the linker that connects the functional entity and the anti-codon, and the zipper-box. The reactive groups X (a carboxylic acid) and Z (an amine) are coupled to the 3'-
10 end of DNA oligo 1 and the 5'-end of DNA oligo 2, respectively. A template sequence to which oligo 1 and oligo 2 would anneal might contain the follow-

ing sequence: 5'-CCGATGCAATCCAGAGGTCG-
GCTGGATGCTCGACAGGTC.

Fig. 23 shows three methods of how the proximity effect can be increased:

(A) Helix stacking, (B) Ligation and (C) Rigid linkers.

(A) Helix stacking. Two building blocks with oligonucleotide-based anti-codons anneal to their respective codons (in the figure, the left building block is a "scaffold" that carries four reactive groups, and the right building block carries a functional entity with e.g. one reactive group, i.e., the latter building block may carry the substituent that will become attached to the scaffold. Double helices tend to stack, especially if the sequence of the opposing ends of the helices has been designed so as to optimize this interaction (for example by the presence of the sequence GGG at the ends of the duplex structures). This stacking tendency will bring the two building blocks into closer proximity, in turn increasing reaction efficiency between the functional entities. If the "substituent-building blocks" have anti-codons with lower melting temperatures than that of the "scaffold-building block", the substituent building block may be removed after its reaction with the scaffold building block, before the next building block is incorporated. In this way, the template region between two reacting building blocks may be kept single stranded, allowing this region to loop out and let the two duplex structures stack during the reaction between the two building blocks.

5 (B) Ligation of building blocks. The anti-codons of two building blocks may be chemically or enzymatically ligated together. Coupling of two anti-codons will increase the annealing efficiency. Therefore, smaller anti-codons can be used if ligated together with the previously incorporated building block. As an example, first add a building block (or just an 20-nucleotide DNA oligo) with a melting temperature of e.g. 60 °C. Then add another building block (e.g.,
10 with a 8-nucleotide DNA anti-codon) with a low melting temperature and therefore only capable of transiently interacting with the template at the ambient temperature. If a DNA ligase is employed, or if the anti-codon can be

ligated to the anti-codon of the first building block chemically, then the second building block will become firmly attached to the template, despite its short length of just 8 nucleotides. Thus, ligation allows the use of shorter anti-codons, which in turn brings the reactive groups into closer proximity.

5

(C) Rigid linkers. By using linkers comprising one or more flexible regions ("hinges") and one or more rigid regions, the probability of two functional entities getting into reactive contact may be increased.

a. Symbol used for building block with a rigid part and two flexible hinges.

10

b. A building block with the characteristics described in (a). The building block contains a continuous oligonucleotide –strand, constituting both the anti-codon (horizontal line), and linker (vertical line) connecting the functional entity (FE) with the anti-codon. Annealing of a complementary sequence to the central part of the linker leads to formation of a rigid double helix; at either

15

end of the linker a single-stranded region remains, which constitutes the two flexible hinges.

Fig. 24 discloses various cleavable linkers. A number of cleavable linkers are shown, as well as the agents that cleave them and the products of the cleavage-reaction. In addition, catalysts including enzymes and ribozymes, may also be used to cleave the linker. Exemplary enzymes are proteases (e.g. chymotrypsin), nucleases, esterases and other hydrolases.

Fig. 25 shows two different ways of templated synthesis by generating a new reactive group. In cases where the reaction of X and Y leads to formation of a new reactive group Z, this may be exploited to increase the diversity of the templated molecule, by incorporating building blocks carrying reactive groups Q that react with Z. Using this approach, the templated molecules may be very compact structures, and thus, this approach describes a method to make highly substituted (functionalized and diverse) libraries of molecules of relatively low molecular weight.

(A) First, a building block carrying a reactive group X and a building block carrying a reactive group Y is incorporated, whereafter X and Y react, leading to the formation of the Z bond. Then a building block carrying a reactive group Q is added, whereafter Z reacts with Q, to form the ZQ bond. In this example, both the reaction of X with Y, and of Z with Q, are reactions that involve simultaneous reaction and cleavage.

(B) First, a building block carrying a reactive group X and a building block carrying a reactive group Y is incorporated, whereafter X and Y react, leading to the formation of the Z bond. Then a building block carrying a reactive group Q is added, whereafter Z reacts with Q, to form the ZQ bond. In this example, the reaction of Z with Q does not involve simultaneous cleavage, wherefore an additional step of linker cleavage is introduced.

Fig. 25, example 1, shows a templated synthesis by generating a new reactive group. The reaction of the functional entities of the first three building blocks leads to formation of two double bonds, which may react with two hydroxylamines carried in by the building blocks added in the latter steps, and leads to formation of an ester, which may react with the hydroxylamine, encoded by a building block. Finally, the linkers are cleaved, generating the templated molecule.

Fig. 26 shows different methods of performing post-templating modifications on templated molecule. After the templating process has been performed, the templated molecules may be modified to introduce new characteristics. This list describes some of these post-templating modifications.

Fig. 27 illustrates one preferred method for selection of template-displaying molecules.

Figure 28 shows the proposed complexes that may form when a reaction step is performed using set-ups that allow for stacking of DNA duplexes.

Figure 29 shows a autoradiography of a polyacrylamide gel analysis of the reaction between building blocks.

- 5 Figure 30 shows the Feuston 3 functional entity as well as the Feuston 5 ligand.

Figure 31 shows the structure of the pentenoyl protected aspartate.

- 10 Figure 32 shows the use of allylglycine building blocks.

Figure 33 shows the autoradiography of a polyacrylamide gel.

- 15 Figure 34 shows an Elisa analysis of the product of the two-step encoding process.

Examples

- 20 In the following examples, building blocks are used which contain a zipper box adjacent to the functional entity. The zipper box sequences are underlined below. The following buffers and protocols are used in the same three examples.

Buffers.

Buffer A (100 mM Hepes pH= 7,5; 1 M NaCl)

Buffer B (20 mM Hepes pH= 7,5; 200 mM NaCl)

25

5'-Labeling with ^{32}P .

Mix 5 pmol oligonucleotide, 2 μl 10 x phosphorylation buffer (Promega cat#4103), 1 μl T4 Polynucleotide Kinase (Promega cat#4103), 1 μl γ - ^{32}P ATP, add H_2O to 20 μl . Incubate at 37°C 10-30 minutes.

PAGE (polyacrylamide gel electrophoresis).

The samples are mixed with formamide dye 1:1 (98% formamide, 10 mM EDTA, pH 8, 0,025 % Xylene Cyanol, 0,025% Bromphenol Blue), incubated at 80°C for 2 minutes, and run on a denaturing 10% polyacrylamide gel. Develop gel using autoradiography (Kodak, BioMax film).

Example 1

The effect of alternating temperature on reaction efficiency in the zipper box system.

DNA-oligos:

X= Carboxy-dT (Glen Research, cat.no. 10-1035)

6= Amino-Modifier 5 (cat. Nr. 10-1905)

AH 316: 5'- 6GTAACAGACCTGTCGAGCATCCAGCT

AH 331: 5'-

CGACCTCTGGATTGCATCGGTGTTACX

AH140: 5'-

AGCTGGATGCTCGACAGGTCAGGTCGATCCGCGTTACCAGTCTTGCC-
TGAACGTAGTCGTCCGATGCAATCCAGAGGTCTG

Experimental.

Mix 10 µl Buffer A, 1 pmol AH 331 (³²P-labelled), 10 pmol AH 316, 5 pmol AH 140, and add H₂O to 50 µl.

Anneal from 80° C to 30° C (-1° C / 30 sek). Then dilute 100 times in buffer B + 50 mM DMT-MM. (Prepared according to Kunishima *et al. Tetrahedron* (2001), 57, 1551) dissolved in ddH₂O.

Incubate at one of 8 different temperature profiles o/n (6 different constant temperatures (15°C; 17,8°C; 22,7°C; 28,3°C; 31,0°C; or 35,0°C; or alternating between 10°C for 5 sec. and 35°C for 1 sec.); or alternating between

20°C for 5 sec. and 45°C for 1 sec). Analyze by 10% urea polyacrylamide gel electrophoresis.

Results.

- 5 The polyacrylamide gel analysis showed that a more efficient reaction results from alternating the temperature between 10 °C and 35 °C, rather than performing the reaction at a constant temperature of 15°C, 17,8°C, 22,7°C, 28,3°C, 31,0°C, or 35,0°C.

10

Example 2

The effect of stacking on reaction efficiency.

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DNA-oligos:

X= Carboxy-dT (cat.no. 10-1035)

Z= Amino-Modifier C6 dT (cat.no. 10-1039)

6= Amino-Modifier 5 (cat.no. 10-1905)

20

AH36: 5'-

CGACCTCTGGATTGCATCGGTCATGGCTGACTGTCCGTCGAATGTGTC-
CAGTTACX

AH38: 5'- AGCTGGATGCTCGACAGGTCCCGATGCAATCCAGAGGTCTG

25

AH51: 5'-

ZGTAACACCTGTGTAAGCTGCCTGTCAGTCGGTACTGACCTGTCTGAG-
CATCCAGCT

AH137: 5'-ACGACTACGTTTCAGGCAAGA

AH138: 5'-

30

TCTTGCCTGAACGTCGTCGTCGATCCGCGTTACCAGAGCTG-
GATGCTCGACAGGTCCCGATGCAATCCAGAGGTCTG

AH139: 5'-CGACCTCTGGATTGCATCGG

AH143: 5'-

CTGGTAACGCGGATCGACCTTCATTTTTTTTTTTTTTTTTTTGGCT-

GACTGTCCGTCGAATGTGTCCAGTTACX

AH 202: 5'-TCTGGATTGCATCGGGTTACX

5 AH 270: 5'- 6GTAACGACCTGTCGAGCATCCAGCT

AH 286: 5'-

AGCTGGATGCTCGACAGGTCAAGTAACAGGTGATCCGCGTTA-

TATCGTTTACGGCATTACCCGTATAGCCGCTAGATGCCCAACCATGACG

GCCCATAGCTTGCGGCTTGC

10 AH 320: 5'-

AGCTGGATGCTCGACAGGTCAAGTCAAGGTGATCCGCGTTACCAGGCC-

CATAGCTTGCGGCTTGCTGCAGTCGATGGACCATGCCTCTTGCT-

GAACGTAGTCGTCCGATGCAATCCAGAGGTGCG

AH 321: 5'-CAAGAGGCAT

15 AH 322: 5'-TCAGGCAAGAGGCATGGTCC

AH 342: 5'-TACTTGACCTGTCGAGCATCGTTACX

AH 343: 5'- 6GTAACCAGCTGCAAGCCGCAAGCTATGGGC

Experimental.

20

Mix buffer A and relevant oligos (see table below).

Experiment	Oligo 1 (³² P- labelled)	Oligo 2	Oligo 3 Template	Oligo 4	Oligo 5	Buffer A	H ₂ O to
1	5 pmol AH 36	10 pmol AH 51	10 pmol AH 38			2 µl	10 µl
2	5 pmol AH 143	10 pmol AH 51	10 pmol AH 138	10 pmol AH 139	10 pmol AH 137	2 µl	10 µl
3	1 pmol AH 202	10 pmol AH 270	5 pmol AH 320			10 µl	50 µl
4	1 pmol AH 36	10 pmol AH 51	5 pmol AH 320			10 µl	50 µl

5	1 pmol AH 202	10 pmol AH 270	5 pmol AH 320	50 pmol AH 321		10 μ l	50 μ l
6	1 pmol AH 36	10 pmol AH 51	5 pmol AH 320	50 pmol AH 321		10 μ l	50 μ l
7	1 pmol AH 202	10 pmol AH 270	5 pmol AH 320	50 pmol AH 322		10 μ l	50 μ l
8	1 pmol AH 36	10 pmol AH 51	5 pmol AH 320	50 pmol AH 322		10 μ l	50 μ l
9	0,2 pmol AH 342	2 pmol AH 343	1 pmol AH 286			2 μ l	10 μ l
10	0,2 pmol AH 342	2 pmol AH 343	1 pmol AH 286	4 pmol AH 356		2 μ l	10 μ l
11	0,2 pmol AH 342	2 pmol AH 343	1 pmol AH 286	4 pmol AH 357	4 pmol AH 358	2 μ l	10 μ l

Anneal from 80°C to 30°C (-1°C/min). Add 0,5 M DMT-MM. (Prepared according to Kunishima *et al. Tetrahedron* (2001), 57, 1551) dissolved in H₂O. to a final concentration of 50 mM. Incubate at 10°C for 5 sec. and then 25°C

5 for 1 sec. Repeat o/n.

Analyze by 10% urea polyacrylamide gel electrophoresis.

Results.

10 In order to test the effect of stacking of DNA duplexes on reaction efficiency, we designed a number of different set-ups of templates and building blocks (see Figure 28). The following conclusions were reached:

15 Figure 28,1 and Figure 29, lane 1: Reference reaction between two building blocks annealed to adjacent sites on the template. As expected an efficient reaction is observed. In this set-up, the two building blocks anneal to the template and thereby form DNA duplexes that can stack onto each other.

Figure 28,2 and Figure 29, lane 2: In this set-up, the two building blocks anneal to adjacent sites on the template. However, the two DNA-duplexes stack onto each other, basically forming one long DNA duplex. This rigid duplex does not allow the two building blocks to bend around the flexible hinge that might otherwise be present at the connection point between the two duplexes (i.e. the position of the nick in the DNA). Consequently, no significant reaction between the two building blocks is observed.

Figure 28,3 and Figure 29, lane 3; and Figure 28,4 and Figure 29, lane 4: Despite the fact that the two building blocks anneal to sites separated by 80 nucleotides, the reaction is still very efficient. We speculate that this is because of stacking, i.e. the intervening 80 nucleotides are looped out as a consequence of this, and therefore, the two functional entities are brought into close proximity.

In the experiment of Figure 29, lane 3 the linker that connects the functional entity to the complementing element is short (5 nucleotides); in Figure 29, lane 4 it is long (35 nucleotides). However, both linker lengths result in an efficient reaction.

Figure 28, 5 and Figure 29, lane 5; and Figure 28, 6 and Figure 29, lane 6: The annealing sites and separation between them are identical to those of the experiment described above (Figure 28, 3 and 4; Figure 29, lanes 3 and 4). In addition, a short oligo (10 nucleotides) has been annealed to the central region of the template. This results in a drastic decrease in reaction efficiency for the building blocks with the short linkers (lane 5); the reaction efficiency of the building blocks with the long linkers is only slightly affected if at all by the annealing of the short oligo. As indicated by the suggested structure of the complexes (Figure 28, 5 and 6), we believe this is because of stacking of the 3 DNA duplexes to generate an "extended" duplex: The short linkers cannot reach across the extended duplex; the long linkers can reach

across the extended duplex structure and the reaction efficiency is not significantly affected.

- 5 Figure 28, 7 and Figure 29, lane 7; and Figure 28, 8 and Figure 29, lane 8:
As immediately above, except that a 20 nucleotide long oligo is annealed to the central region of the template. In this case none of the linkers (short or long) can reach across the extended duplexes, and as a result no or little reaction is observed.
- 10 Figure 28,9 and Figure 29, lane 9; Figure 28,10 and Figure 29, lane 10; and Figure 28,11 and Figure 29, lane 11: In these experiments the building blocks are oriented the "other way", i.e. the linker connecting the complementing element and the functional entity is near the ends of the template. Additionally, the complementing element of the left building block contains a
- 15 5-nucleotide sequence that is complementary to other right end of the template. As a result, the building block should be capable of circularizing the template, as depicted in Figures 27, 9-11. These circular structures should also be stabilized by an extended duplex structure across the ends of the template. In the experiments of lanes 10 and 11, a short oligo (10 nucleotides) or two longer oligos (each 20 nucleotides) are annealed to the central
- 20 region. This has no effect on the reaction efficiency, in correlation with the proposal that the building blocks stack onto each other through a circularization of the template, thereby bringing the functional entities into close proximity.

25

Example 3

Single step transfers of functional entities.

30 DNA-Oligos:

7=Thiol-Modifier C6 S-S (Glen Research, cat.no.10-1936)

Z=Amino-Modifier C6 dT (10-1039)

P= PC Spacer (10-4913)

AH136: 5'- AGCTGGATGCTCGACAGGTCTCTTGCCTGAACGTAGTCG-
TCCGATGCAATCCAGAGGTCG

5 AH 174: 5'-TACGTTTCAAGCAAGAGT6CCAGTTAC7

AH 190: 5'- ZGTAACACCTGPTGACCTGTCGAGCATC

Experimental:

10 Loading of NHM on the DNA-oligo:

Dry 10 nmol DNA oligo (AH174) and then resuspended in 50 μ l 100 mM DTT (1,4-Dithio-L-Threitol D-9760 Sigma) in 50 mM Phosphate buffer pH=8. Incubate at 37°C for 1 hour.

Purification on Microspin G-25 (Amersham Biosciences, 27-5325-01).

15 Add 50 μ l 200 mM NHM (N-Hydroxymaleimide Fluka 55510) and incubate at 25°C for 2 hours.

Purification on Microspin G-25 equilibrated in H₂O.

20 Loading of building blocks (4-pentenoic-acid, β -ala-Boc or CH₃COOH) on the NHM-DNA-oligo:

Mix 50 μ l 100 mM EDC and 50 μ l 100 mM building block. Incubate at 25°C for 30 minutes.

Then mix 500 pmol NHM-DNA-oligo (AH174-NHM) and 10 μ l of the EDC/building block mix from above. Add 100 mM MES pH=6 to 20 μ l. Incubate at

25 25°C for 5 minutes.

Purification on Micro Bio-Spin Chromatography Columns P6 (Bio-Rad 732-6221) equilibrated in 100 mM MES pH=6.

Transfers:

30 Mix 350 pmol AH136, 300 pmol AH190 and 500 pmol building block loaded AH174. Add Buffer A to 50 μ l.

Anneal from 60°C to 25°C (-1°C/ 30 sec.)

Incubate at 10°C for 5 sec. and then 25°C for 1 sec. Repeat o/n.
Purification on Micro Bio-Spin Chromatography Columns P6 equilibrated in H₂O.

5 **Results:**

The transfers were analyzed by MS, see table below. Transfer efficiencies of 20-34% were observed.

Transfer efficiency		
4-pentenoic-acid	β-ala-Boc	CH ₃ COOH
33-34%	20-23%	29-33%

10

Example 4

Multistep transfer of functional entities to a scaffold oligonucleotide

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In this example three functional entities are transferred to an amino modified scaffold oligo by a three step reaction, and analyzed by a denaturing acrylamide gel using radio labelling.

20

Loading of functional entities on modified oligonucleotides to create building blocks.

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5 nmoles of three carboxylic acid modified building block oligos [AH 155; 5'CTG GTA ACG CGG ATC GAC CTG TTA CT-COOH 3'; AH 272 5'ACG ACT ACG TTC AGG CAA GAG TTA CT-COOH 3' and AH 202 5'-TCT GGA TTG CAT CGG CTG TTA CT-COOH 3'] (all oligonucleotides described ordered from DNA technology, Aarhus, Denmark) one from each of the three positions corresponding to the template were loaded with β-Alanine methyl ester coupled to allylglycine n-Boc followed by Boc deprotection (β-AlaOMe AG). The loading was done by incubating each of the oligos with 10 mM β-

AlaOMe AG, 75 mM DMT-MM in 150 mM Hepes-OH buffer, pH 7,5 to a final volume of 50 μ l at 25°C shaking overnight. Then adding 5 μ l 1 M NH_4 -acetate, incubated at 25°C for 10 min, then spin column purified with ddH₂O equilibrated columns (Micro Bio-Spin chromatography columns P-6, Bio-
5 Rad). The deprotection of the methyl group protected acid was done by adding 0,5 μ l 2M NaOH to the oligos and incubating for 10 min at 80°C. Lastly the oligos were spin column purified and loadings confirmed by mass spectrophotometry.

10 Transfers of functional entities to scaffold oligo.

In order to be able to analyze the functional entity transfers using acrylamide gel analysis, the scaffold oligo [MDL251 5' amino-C6 dT-ACC TGT CGA GCA TCC AGC T 3'] was radioactively labelled in the 3' end. 50 pmol of the oligo
15 was labelled with 10 μ l ddATP α P32 (Amersham Biosciences) by adding 4 μ l 10X NEbuffer 4, 4 μ l 10X CoCl₂ and 35 units of terminal deoxynucleotide transferase (New England Biolabs) and water to a final volume of 40 μ l. Mixture incubated at 37°C for 1 hour. Labeled oligo purified using ddH₂O equilibrated spin column.

20

12,5 pmol of the labeled scaffold oligo, 125 pmol loaded building block oligo AH 202, corresponding to position three on the template and 62,5 pmol template [AH 154 5' AGC TGG ATG CTC GAC AGG TCA AGT AAC AGG TCG ATC CGC GTT ACC AGT CTT GCC TGA ACG TAG TCG TCC GAT GCA
25 ATC CAG AGG TCG 3'] was incubated in a final volume of 45 μ l containing 20 mM Hepes-OH pH 7,5, 200 mM NaCl buffer. The oligos were annealed by heating to 80°C and slowly going down to 20°C (1°/min) using a thermocycler (Eppendorf, Mastergradient) Following the annealing 5 μ l 0,5M DMT-MM was added. Sample crosslinked, see figure 32 overnight cycling at 10°C
30 10 sec/35°C 1 sec.

The sample was spin column purified and the crosslinked product cleaved to give first transfer of β -Ala to scaffold oligo amine by adding 10 μ l 25 mM I2 dissolved in 1:1 tetrahydrofuran:H₂O and incubated at 37°C for 1,5 hours. Followed by addition of 1,5 μ l 1 M β -mercapotethanol and then purified with two equilibrated spin columns. The sample was completely dried down and oligos redissolved in 30 μ l ddH₂O. Transfer 2, oligo AH 272 and transfer 3, AH 202 were done in the exact same way as just described including the annealing, crosslinking and cleavage. For each remaining round adding same amount of building block oligo, 125 pmol.

Samples for analysis were taking out along the way, before and after crosslinking for the three transfers, which were analyzed on a 10% acrylamide denaturing gel, see fig 33. As can be seen, crosslinking efficiency (step 1) was approximately 50% (Figure 33, lane 1). This was followed by an almost 100% efficient cleavage (lane 2), which results in the transfer of the β -Ala moiety onto the scaffold. This is followed by the crosslinking/cleavage of step 2 and 3 (lanes 3+4, 5+6) to generate the final product on the scaffold oligo. The product thus contains the three transferred β -Ala moieties.

Example 5

Two-step transfer and functional analysis by ELISA.

In this example two entities are transferred to a scaffold oligo by a two-step reaction to produce a ligand, Feuston 5 (see Figure 30) that binds to the α V β 3 integrin receptor. The product of the two-step process was analyzed by Elisa.

Loading of functional entities on modified oligonucleotides to create building blocks.

Two building block oligos were used, AH 155 (see above) loaded with Feuston 3 allylglycine. Feuston 3 is a derivative of the Feuston 5 ligand see fig 30 (F3OMeAG) and AH 272 (see above) loaded with glycine allylglycine (GlyO-

MeAG) according to the above protocol (example Xa) for loadings of allylglycine functional entities to carboxylic acid modified oligos. 10 nmoles of each was loaded in two reactions each.

To create the Feuston 5 ligand aspartate is also needed. Therefore aspartate which was loaded as a pentenoyl (amine) and methyl (carboxylic acid) protected functional entity see Figure 31, to an amino modified scaffold oligo [AH 270 ;5' amino-GTA ACG ACC TGT CGA GCA TCC AGC T 3']. The loading was done by mixing 25 µl 150 mM EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, Fluka), 25 µl NHS (N-hydroxysuccinimide, Sigma) and 5 µl 100 mM of the pentenoyl protected aspartate functional entity, all reagents were dissolved in N,N-dimethylformamide, DMF. Incubated at 25°C for 40 min. To this mixture 5 nmol of the scaffold oligo, AH 270 resuspended in 30 µl 150 mM Hepes-OH pH 7,5 was added and this incubated shaking over night at 25°C. The amine pentenoyl protection group was deprotected by adding 20 µl 25 mM I2 dissolved in 1:1 tetrahydrofuran: water and incubated at 37°C for 2 hours. Followed by spin column purification, and loading confirmed by mass spectrum analysis.

20 Transfers of functional entities to scaffold oligo.

The transfers were done in the same manner as described above, but using larger amounts of oligo to ensure there being enough ligand created to give a sufficient signal in the ELISA. For the first round the following amounts were used: 850 pmol loaded scaffold oligo; AH 270, 7500 pmol loaded building block oligo; AH 272 and 3250 pmol template oligo AH 140 [5' AGC TGG ATG CTC GAC AGG TCA GGT CGA TCC GCG TTA CCA GTC TTG CCT GAA CGT AGT CGT CCG ATG CAA TCC AGA GGT CG 3']. The second round, adding 7500 pmol loaded building block oligo AH 155 for a transfer. The created Feuston 5 ligand on the scaffold oligo still had a methyl group protected acid on the aspartate, which was deprotected just as described before. By adding 0,5 µl 2 M NaOH to the oligos and incubating at 80°C for

10 min. The sample this time though was pH calibrated with 0,5 µl 2 M HCl and was now ready for the ELISA analysis.

ELISA assay

5

Maxisorb plates (Nunc Immunomodule U8 Maxisorp. Biotecline) were coated with αVβ3 integrin receptor 0,1 µg/well in PBS over night at 4°C. The wells were blocked with 300 µl blocking buffer containing PBS, 0.05% Tween 20 (Sigma), 1% BSA (Sigma), 0.1 mg/mL herring sperm DNA (Sigma), for 3
10 hours at room temperature. Wells were washed 5 * 300 µl using wash buffer containing PBS, 0.05% Tween 20, 1% BSA. The sample prepared above containing the displayed Feuston 5 ligand on a scaffold oligo was added to a well, control for the experiment being a 20 mer oligo loaded with the RGD peptide, a well known and well described ligand for this integrin receptor
15 (loaded according to above described method for the pentenoyl and methyl protected aspartate functional entity). The incubation with these ligands was done in ligand binding buffer containing PBS, 1 mM MnCl₂, 1 mg/mL BSA at room temperature for one hour. Washed in washing buffer 5 * 300 µl. Incu-
20 bated with 100 µl horseradish peroxidase-streptavidine (Endogen) diluted 1:10000 times in wash buffer, incubated for one hour at room temperature. Washed again in 5 * 300 µl wash buffer. 100 µl 3, 3', 5,5'-tetramethylbenzidine hydrogenperoxidase (TMB substrate, Kem-en-tec) added and incubated at room temperature until color development. 100 µl 0,2 M sulphuric acid
25 added, color measured at 450 nm, see figure 34. As can be seen the Feuston 5 ligand generated by the two-step encoding procedure is active and binds the integrin receptor with relatively high efficiency.

Claims

1. A method for the manufacture of a library of complexes comprising templated molecules, said method comprises the steps of
- 5 a) providing a plurality of different templates comprising a number of coding regions and a reactive group, wherein each coding region of a specific template specifies a unique codon,
 - b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of
10 each building block complements a unique codon of a template, and the functional entity comprises at least one reactive group,
 - c) contacting the plurality of different templates with a subset of the plurality of different building blocks, said subset having anti-codons which complement the unique codons of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codons to the unique codons of the templates,
15
 - d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,
 - e) contacting under conditions allowing specific hybridisation, the plurality
20 of different templates harbouring the nascent templated molecules with a further subset of the plurality of building blocks, said subset having anti-codons complementary to the unique codons of a coding region in the vicinity of the coding region harbouring the nascent templated molecules,
 - 25 f) allowing the functional entities of the subset of further building blocks to form a chemical connection to the nascent templated molecules,
 - g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,
 - 30 h) optionally repeating steps e) through g),
 - i) obtaining a templated molecule attached via the linker one or more building blocks to the template which directed the synthesis thereof.

2. The method according to claim 1, wherein the reactive group of step a) comprised by the template is covalently attached to the template.
- 5 3. The method according to claim 1, wherein the reactive group of the template is non-covalently attached to the template.
4. The method according to claim 3, wherein the reactive group of the template is covalently attached to a complementing element hybridised to the
10 template.
5. The method according to claim 3 or 4, wherein the reactive group of the template is part of a building block.
- 15 6. The method according to any of the claims 3 to 5, wherein the building blocks harbouring the reactive group of step a) and the subset of building blocks contacted with the templates in step c) are positioned next to each other.
- 20 7. The method according to any of the preceding claims, wherein the individual coding regions of the plurality of templates are positioned next to each other in a linear sequence.
8. The method according to claims 1 to 6, wherein the template is
25 branched.
9. The method according to any of the claims 1 to 8, wherein coding regions are separated by a spacer group.
- 30 10. The method according to claim 9, wherein the spacer group identifies the neighbouring coding region or unique codon.

11. The method according to any of the preceding claims, wherein the number of coding regions is 3 to 100.
12. The method according to any of the preceding claims, wherein the number of unique codons within a coding region is between 1 and 10,000.
13. The method according to any of the preceding claims, wherein each unique codon is a sequence of 3 to 100 nucleic acid monomers.
14. The method according to claim 13, wherein each unique codon comprises a sequence of 8 to 30 nucleic acid monomers.
15. The method according to any of the preceding claims, wherein the individual unique codon:anti-codon hybrids within a specific coding region have a similar annealing temperature.
16. The method according to any of the claims 1 to 14, wherein the individual unique codon:anti-codon hybrids within a specific coding region have a different annealing temperature.
17. The method according to any of the preceding claims, wherein the functional entity of a building block comprises a reactive group capable of forming a connection to a reaction partner of another functional entity or nascent templated molecule.
18. The method according to any of the preceding claims, wherein the functional entity of a building block comprises a reactive group capable of forming a connection to a reactive group of another functional entity or nascent templated molecule through a bridging fill-in group.
19. The method according to any of the preceding claims, wherein the linker is attached to the anti-codon oligonucleotide at a central area thereof.

20. The method according to any of the preceding claims, wherein the anti-codon and the linker is a contiguous linear oligonucleotide.
- 5 21. The method according to any of the preceding claims, wherein the linker is attached to the functional entity through a reactive group capable of forming a connection to another functional entity or a nascent templated molecule.
- 10 22. The method according to claim 21, wherein the linker is capable of being cleaved simultaneously with the formation of the connection.
23. The method according to any of the preceding claims, wherein the reactive groups involved in the formation of the connection between functional entities or a functional entity and a nascent templated molecule are reactions partners.
- 15 24. The method according to any of the preceding claims, wherein the subset in steps c) comprises building blocks having anti-codons which form hybrids with unique codons in a coding region neighbouring the reactive group of the template.
- 20 25. The method according to any of the preceding claims, wherein the subset in step e) comprises building blocks having anti-codons which form hybrids with unique codons in a coding region neighbouring the building block harbouring the nascent templated compound.
- 25 26. The method according to claims 24 or 25, wherein the subset is formed by adding the building blocks separately.
- 30

27. The method according to any of the claims 24 or 25, wherein the subsets in steps c) or e) are formed by directing the annealing temperature of the individual building blocks.
- 5 28. The method according to any of the preceding claims, wherein the anti-codon of a building block with a functional entity is ligated to the anti-codon of a building block harbouring a nascent molecule prior to establishing the connection between the functional entity and the nascent molecule being prepared.
- 10 29. The method according to any of the preceding claims, wherein building blocks intended to interact with each other each are provided with a part of a molecule pair being capable of reversible interaction.
- 15 30. The method according to claim 29, wherein the one part of the molecule pair is present on the linker, close to the functional entity or nascent templated molecule.
- 20 31. The method according to claims 29 or 30, wherein the one part of the reversible interacting molecule pair of a first building block is an oligonucleotide and the other part of the reversible interacting molecule pair of a second building block intended to interact with the first building block is a complementing oligonucleotide.
- 25 32. The method according to the preceding claims 29 to 31, wherein the annealing temperature of an interacting molecule pair is lower than the annealing temperatures for the unique codon:anti-codon hybrids of the involved building blocks.
- 30 33. The method of claim 32, wherein the annealing temperature of the reversible interacting molecule pair is below room temperature but above 5°C.

34. The method according to claim 33, wherein the annealing temperature is between 10°C and 20°C.
35. The method according to any of the preceding claims, wherein the linker is rigid and attached the anti-codon through a molecular hinge.
36. The method according to claim 35, wherein the rigid linker is a double stranded oligonucleotide.
37. The method according to claim 35 or 36, wherein the molecular hinge is a single stranded region of the building block.
38. The method for the manufacture of a library according to claim 1, wherein the complexes obtained comprise templated molecules attached to the template which templated the syntheses thereof via a single building block.
39. The method according to claim 1, comprising the further step of connecting the templated molecule with the template which directed the syntheses thereof, or a complementing template, via a covalent link.
40. The method according to claim 39, wherein the covalent link is selectively cleavable to provide for a release of the templated molecule.
41. The method according to claim 1, wherein the templated molecules of the library complex are polymers.
42. The method according to any of the claims 1 to 41, wherein the optional cleavage of some or all of the linkers of step g) are not performed.
43. The method according to claim 42, comprising the further step of cleaving all but one linker after the formation of the templated molecule.

44. The method according to any of the preceding claims, wherein the anti-codons following the cleavage of the linker attached thereto, remain hybridised to the unique codons.
- 5 45. The method according to claim 44, wherein the anti-codons attached to the templates are ligated together to create a complementary template.
- 10 46. The method according to claim 1, comprising the further step of transferring the templated molecule to an anchorage point on the template, or a sequence complementing the template, to establish an effective chemical connection.
- 15 47. The method according to claim 46, wherein the complementing sequence has a higher annealing temperature than one or more of the building blocks.
- 20 48. The method according to claim 1, comprising the further step of connecting the templated molecule with a complementary template via a covalent link.
- 25 49. The method according to claim 48, wherein the template is covalently connected to the complementing template.
50. The method according to claim 48, wherein the covalent link is selectively cleavable to provide for a separation of the templated molecule from the complementary template.
- 30 51. A library of complexes obtainable according to any of the claims 1 to 45.

52. A method of enriching a library of complexes comprising templated molecules with respect to a predetermined activity, said enrichment method comprising the steps of:

- 5 i) establishing a first library of complexes comprising templated molecules, said library being obtainable according to any of the claims 1 to 50,
- ii) exposing the library to conditions enriching the library with complexes having the predetermined activity,
- iii) amplifying the complexes of the enriched library,
- 10 iv) optionally, repeating step ii) to iii), and
- v) obtaining an enriched library having a higher ratio of complexes comprising templated molecules with the predetermined activity.

53. The method of claim 52, wherein step iii) comprises a 10^1 to 10^{15} -fold amplification.

54. The method of claim 52, wherein the steps ii) and iii) are repeated at least 2, 3, 5 times, such as at least 10 times, such as at least 15 times.

20 55. The method of claim 52, further comprising a step of identification of the complexes having the predetermined activity.

56. The method of claim 52, wherein the identification is conducted by analysing the template and/or complementary template associated with the molecule.

57. The method of claim 52, wherein the conditions enriching the library comprises contacting a binding partner to the templated molecules of interest.

30 58. The method according to claim 57, wherein the binding partner being directly or indirectly immobilised on a support.

59. The method according to claim 52, wherein the enrichment is conducted by screening for complexes having an affinity for – or an effect on – a target molecule or a target entity.

5

60. The method according to claim 52, wherein the enrichment is conducted by selection for catalytic activity.

10

61. The method of claim 52, wherein the conditions enriching the library involves any one or more of electrophoretic separation, gelfiltration, immunoprecipitation, isoelectric focusing, centrifugation, and immobilization.

15

62. The method of claim 52, wherein the conditions enriching the library comprises providing cells capable of internalising the templated molecule, or performing an interaction with the templated molecule having the desired predetermined activity.

20

63. The method according to claim 52, wherein the amplification of the complexes of the enriched library comprises the steps of

- A. contacting the library of complexes with amplification means,
- B. amplifying the templates or the complementing templates, and
- C. conducting the method according to any of the claims 1 to 50 using the amplification product of step B as templates.

25

64. A method for the manufacture of a complex of a templated molecule attached to the template which directed the synthesis thereof, said method comprises the steps of

30

- a) providing a template comprising a number of coding regions and a reactive group, wherein each coding region specifies a unique codon,
- b) providing a plurality of different building blocks, each building block comprising an anti-codon, a functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon of

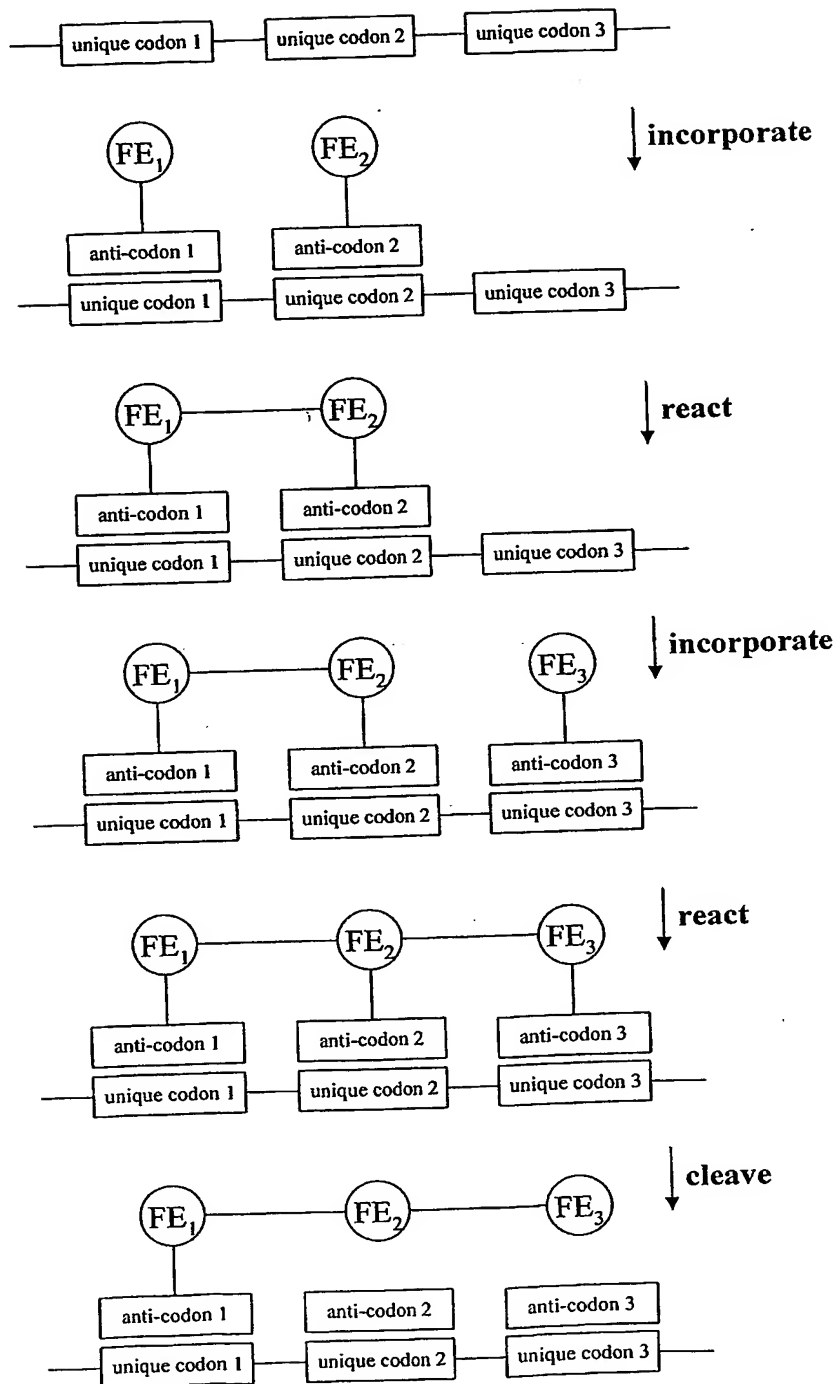
each building block complements a unique codon of the template, and the functional entity comprises at least one reactive group,

- 5 c) contacting the template with a building block having an anti-codon which complements the unique codon of a specific coding region, said contacting being performed under conditions which allow specific hybridisation of the anti-codon to the unique codon of the templates,
- d) reacting the reactive group of the template and the reactive group of the building block to obtaining a chemical connection,
- 10 e) contacting under conditions allowing specific hybridisation, the template harbouring the nascent templated molecule with a further building block having an anti-codon complementary to the unique codon of a coding region in the vicinity of the coding region harbouring the nascent templated molecule,
- 15 f) allowing the functional entity of the further building block to form a chemical connection to the nascent templated molecule,
- g) optionally, cleaving one or more of the linkers, provided that at least one linker remains to connect the nascent templated molecule with the template which directed the synthesis thereof,
- 20 h) optionally repeating steps e) through g),
- i) obtaining a templated molecule attached via the linker of one or more building blocks to the template which directed the synthesis thereof.

60. A method for preparing a templated molecule, comprising the further step of claim 59 of cleaving the linker(s) of the one or more building blocks to
25 release the templated molecule.

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Fig. 1



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Fig. 2

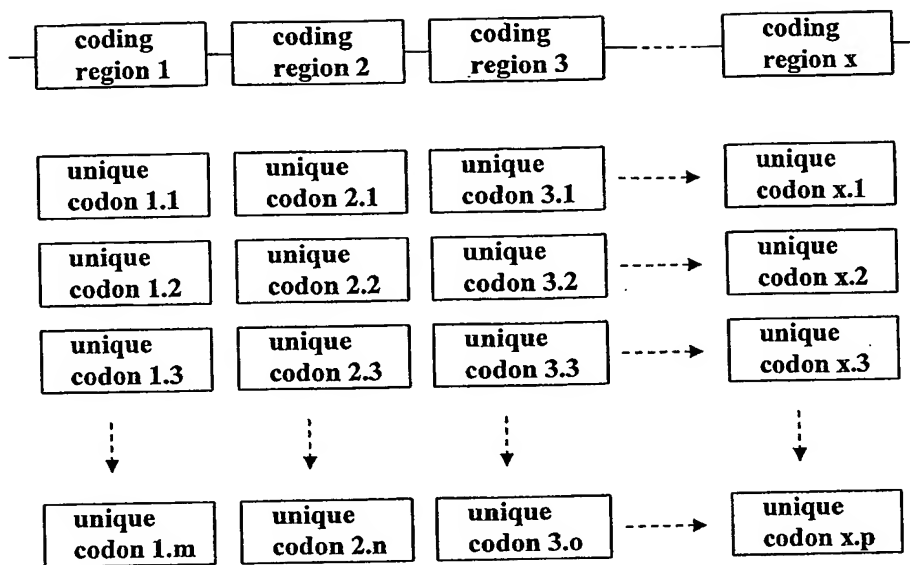


Fig. 3. An oligonucleotide-based building block. Example of coding region design, allowing for high building block diversity.

A.

Coding region	sequence	number of unique codons
1	XXXXXXATATTTXXXXXX	1024
2	XXXATTTTAXXXXXXXXX	1024
3	XTAATTTXXXXXXXXXX	1024
4	XXATXXATXXATXXXX	1024
5	GCCCGATTAAAXXCCG	4
6	XAXAXTTXTTXXXGGG	128

X = G or C

B.

Codon 1	GCGCGATATTTGGGCC
Anti-codon 1	CGCGCTATAAACCCGG
Codon 6	GAGAGTTCTTCGCGGG
Anti-codon 6	CTCTCAAGAAGCGCCC

Fig. 4. Building blocks.

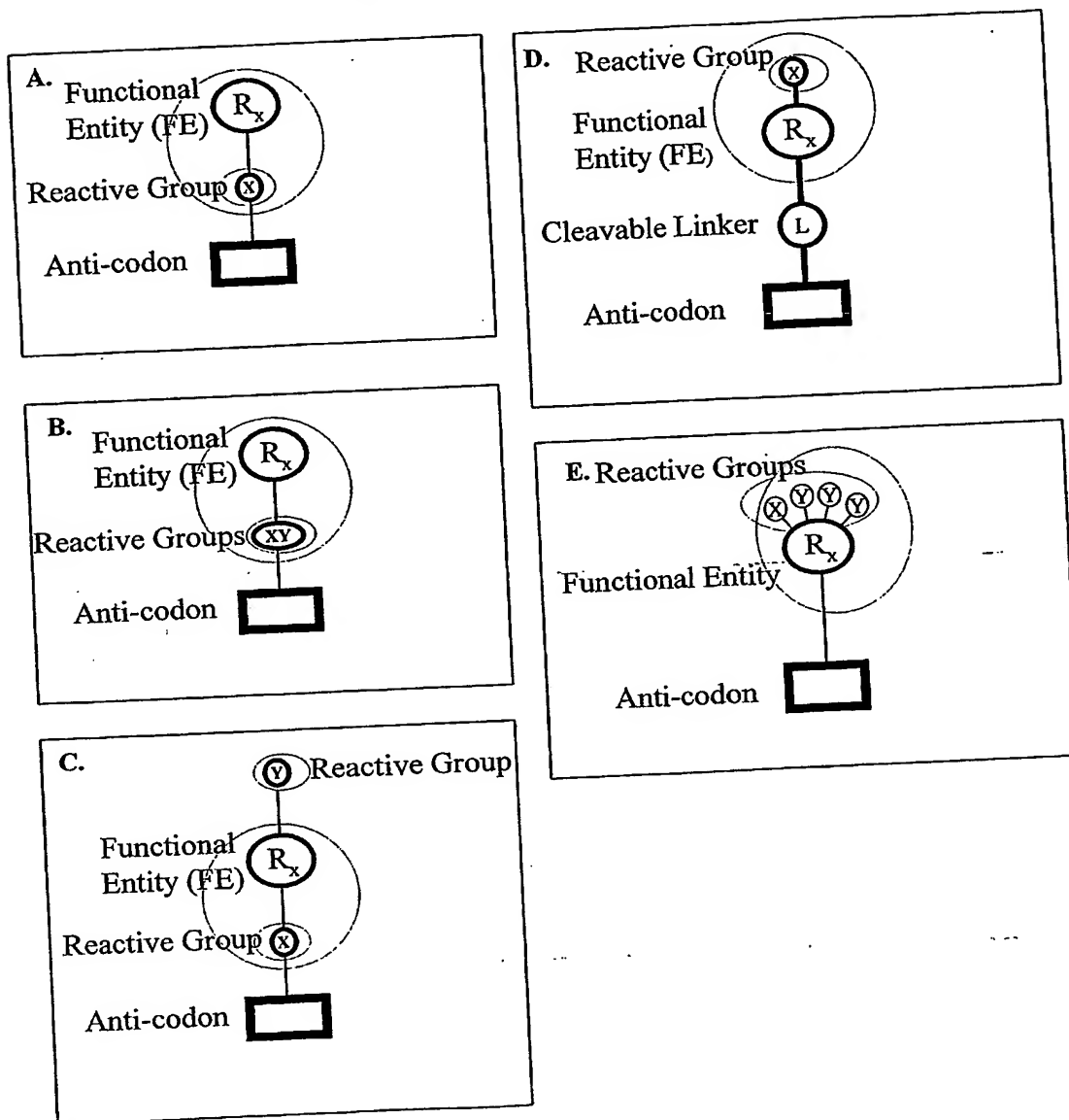


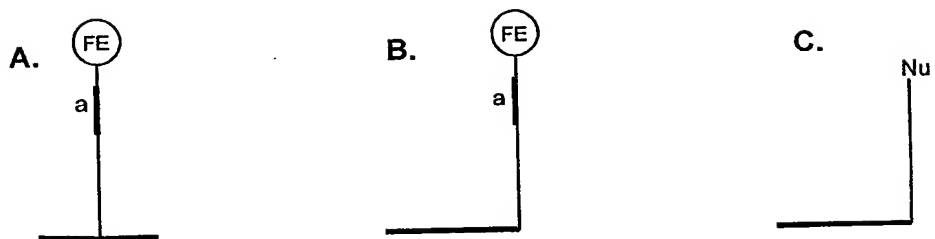
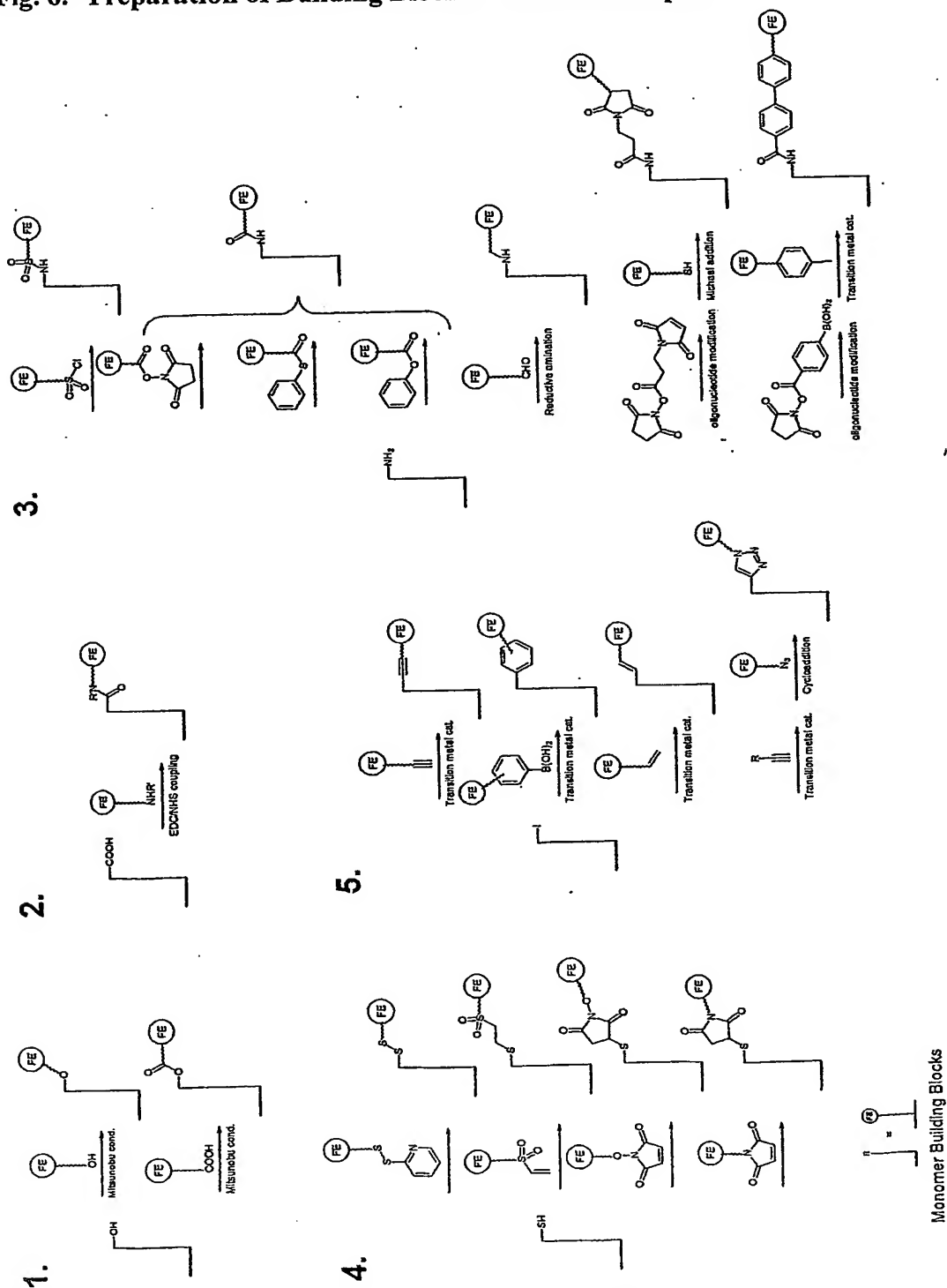
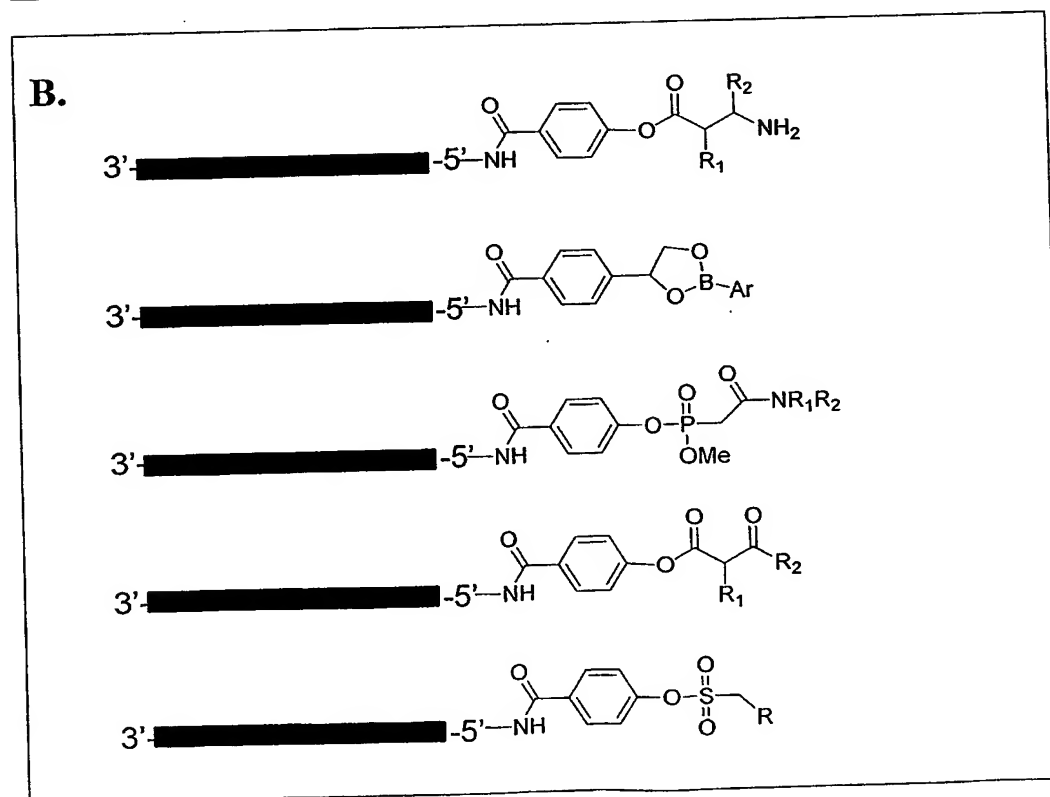
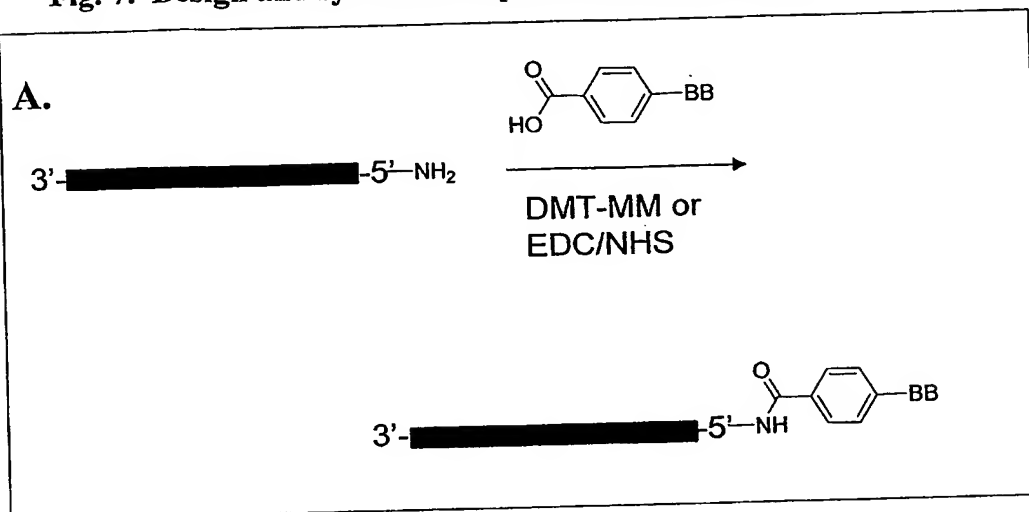
Fig. 5. Exemplary monomer Building Blocks.

Fig. 6. Preparation of Building Blocks. General examples



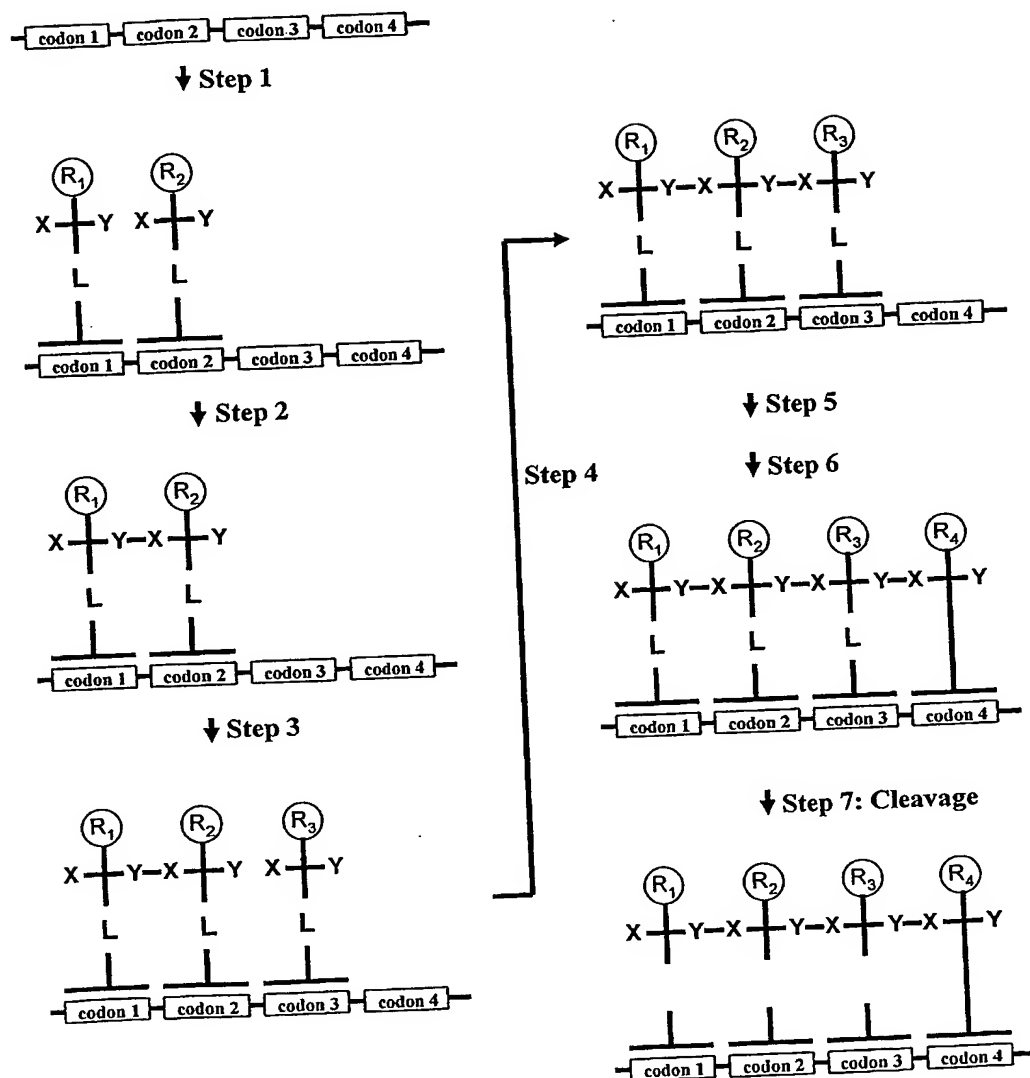
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Fig. 7. Design and synthesis of specific building blocks

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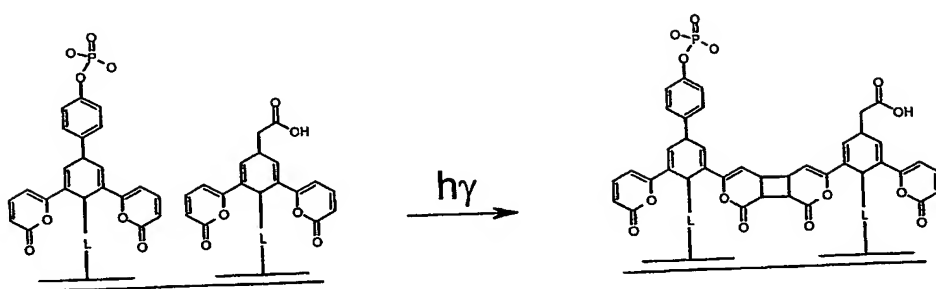
Fig. 8. Templated synthesis of a polymer.



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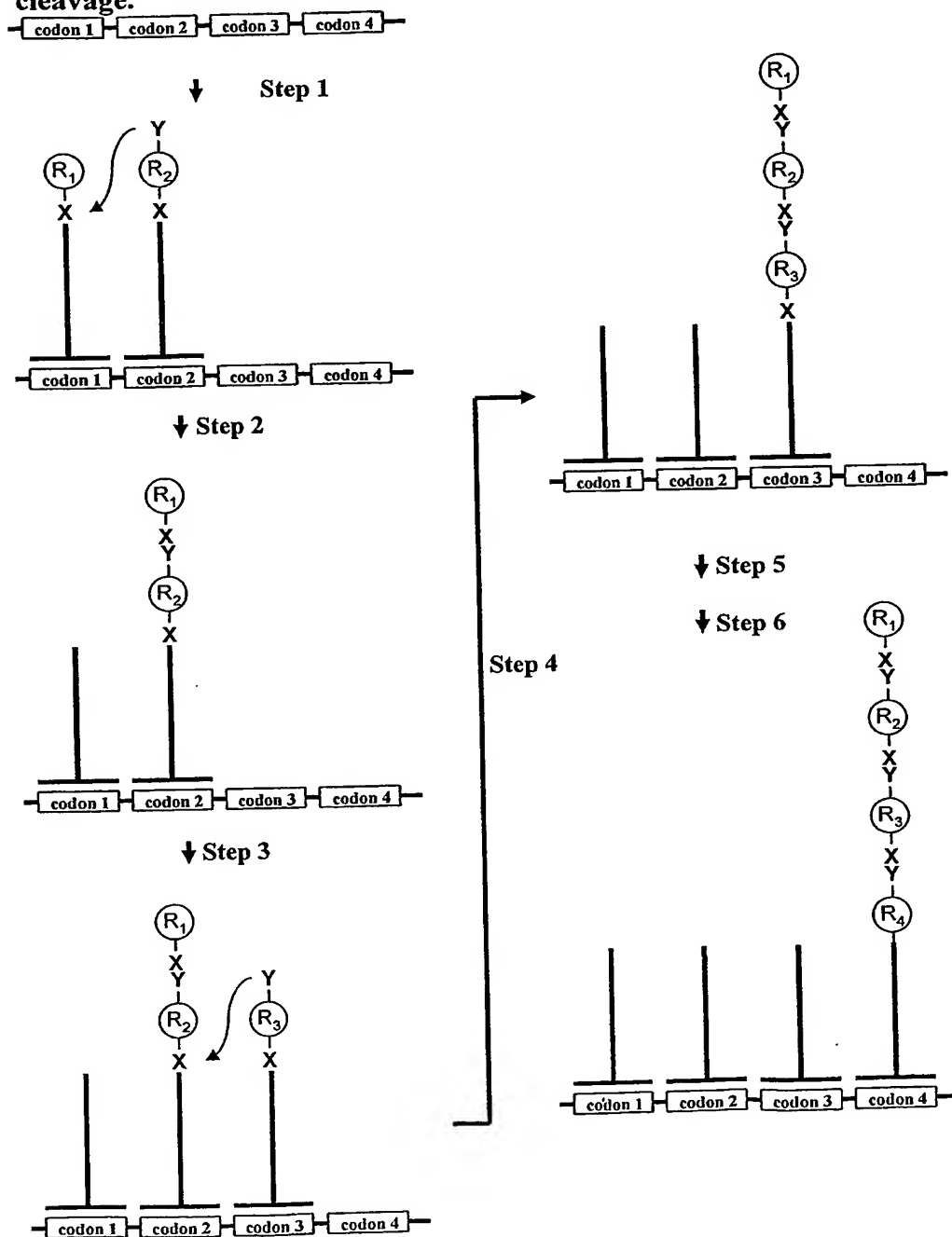
Fig. 8, example 1. Light-induced reaction between symmetric building blocks: Coumarin derivatives.

A



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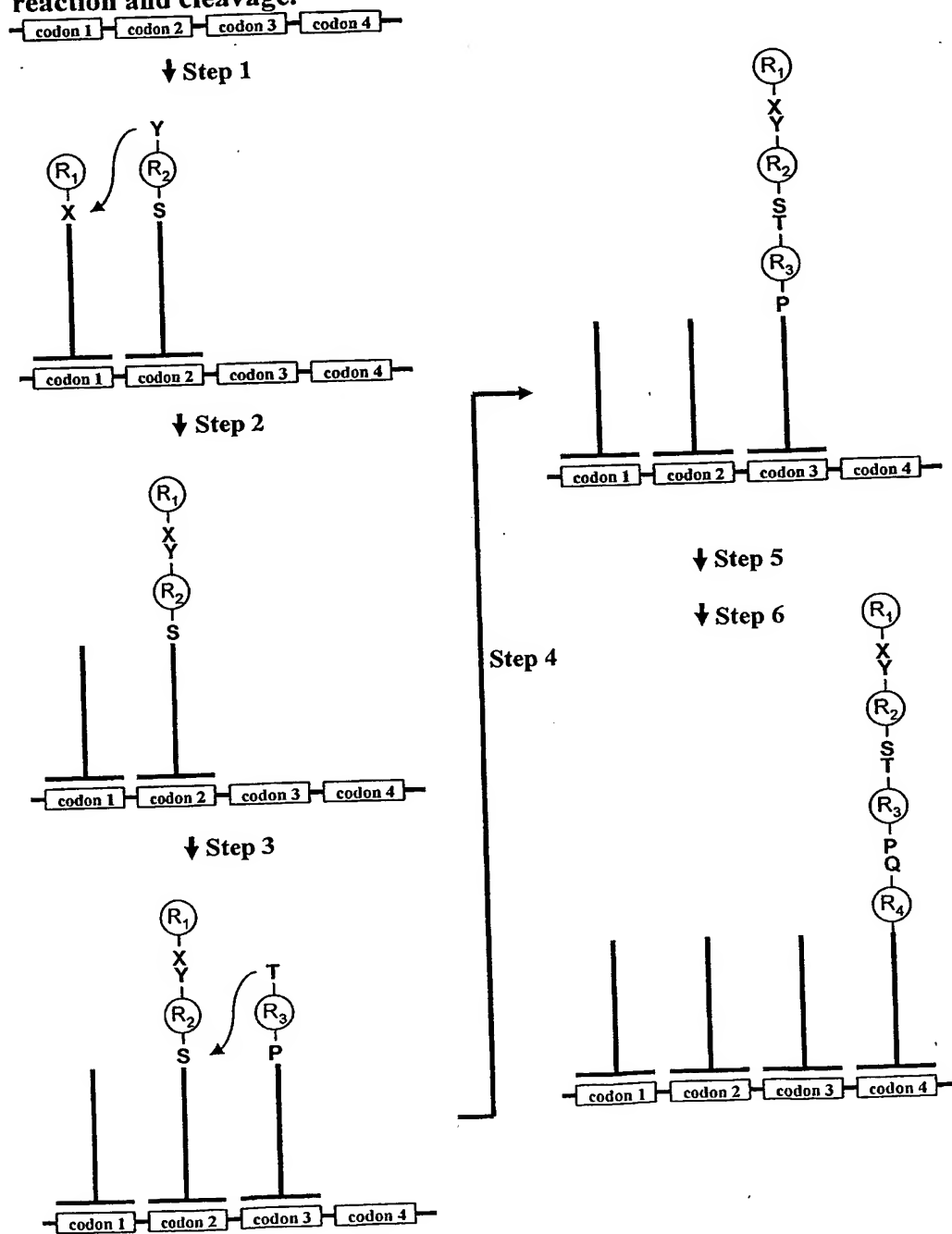
Fig. 9. Templated synthesis of a polymer by simultaneous reaction and cleavage.



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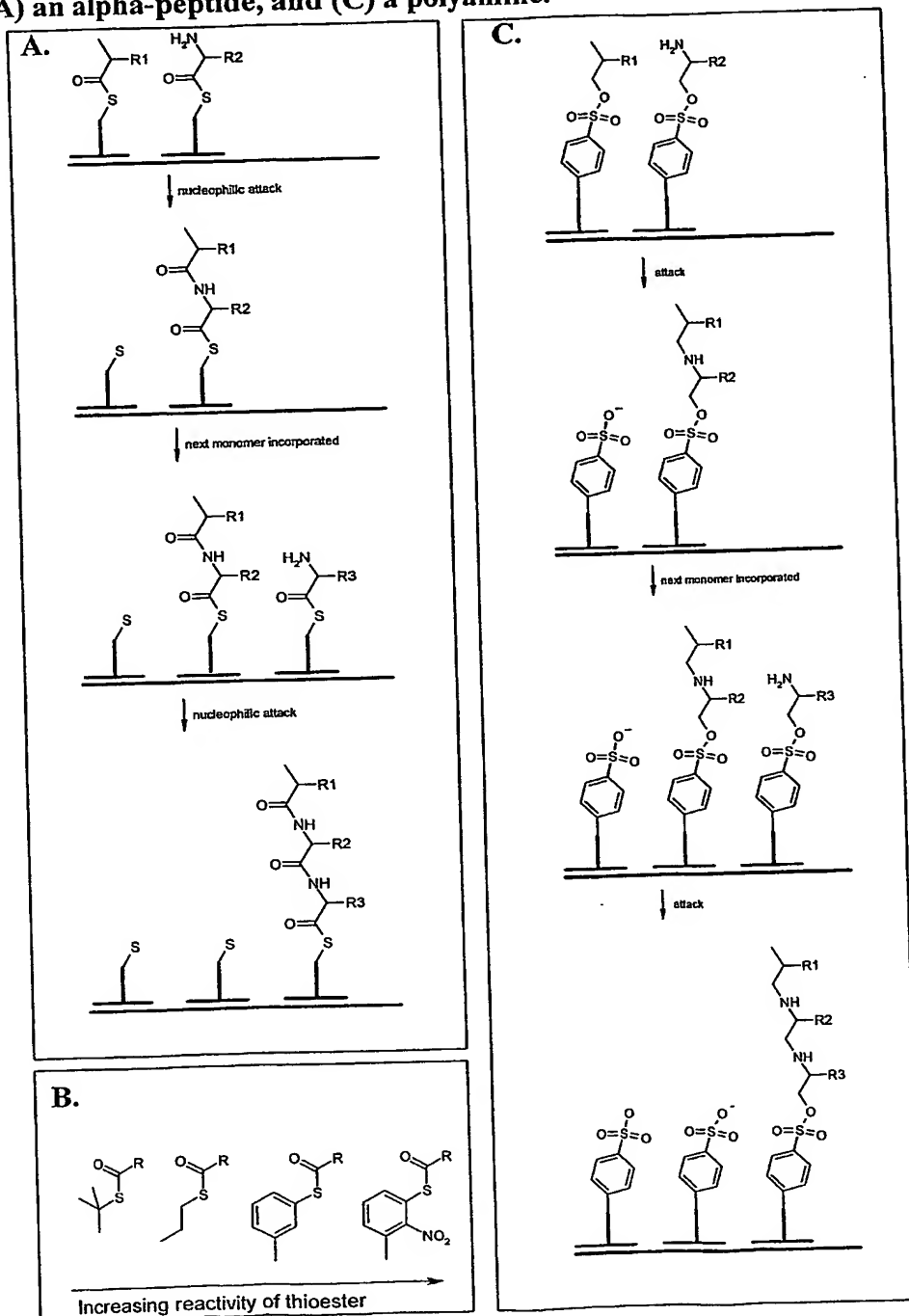
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Fig. 10. Templatd synthesis of a mixed polymer by simultaneous reaction and cleavage.



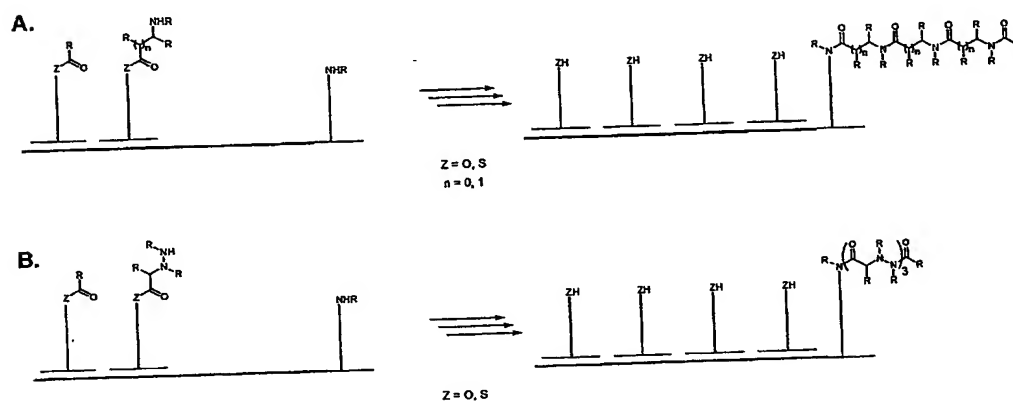
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Fig. 10, example 1. Simultaneous reaction and cleavage: Formation of (A) an alpha-peptide, and (C) a polyamine.



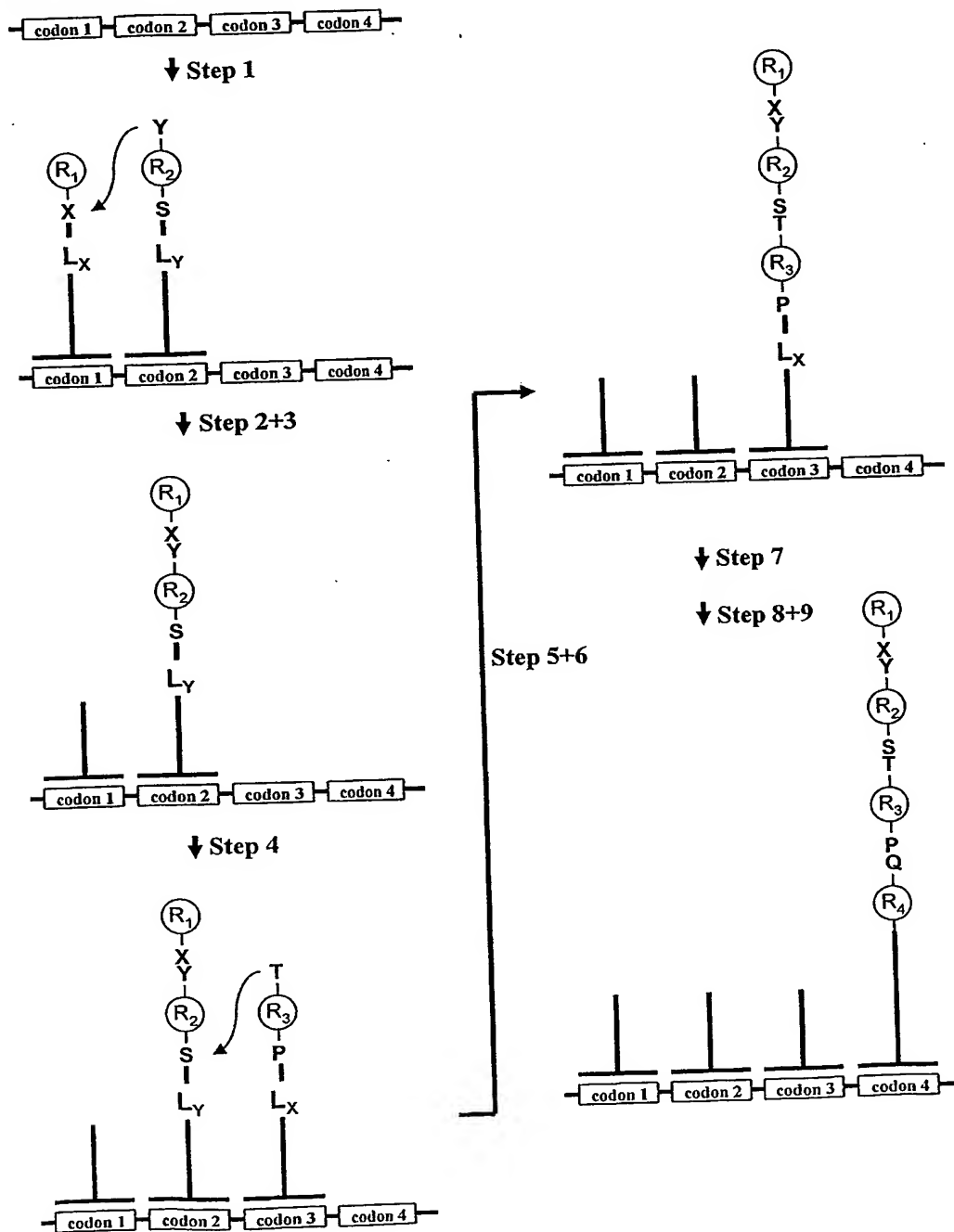
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Fig. 10, example 2. Simultaneous reaction and cleavage: Formation of (A) a peptoid or an alpha- or beta-peptide, and (B) a hydrazino peptide.



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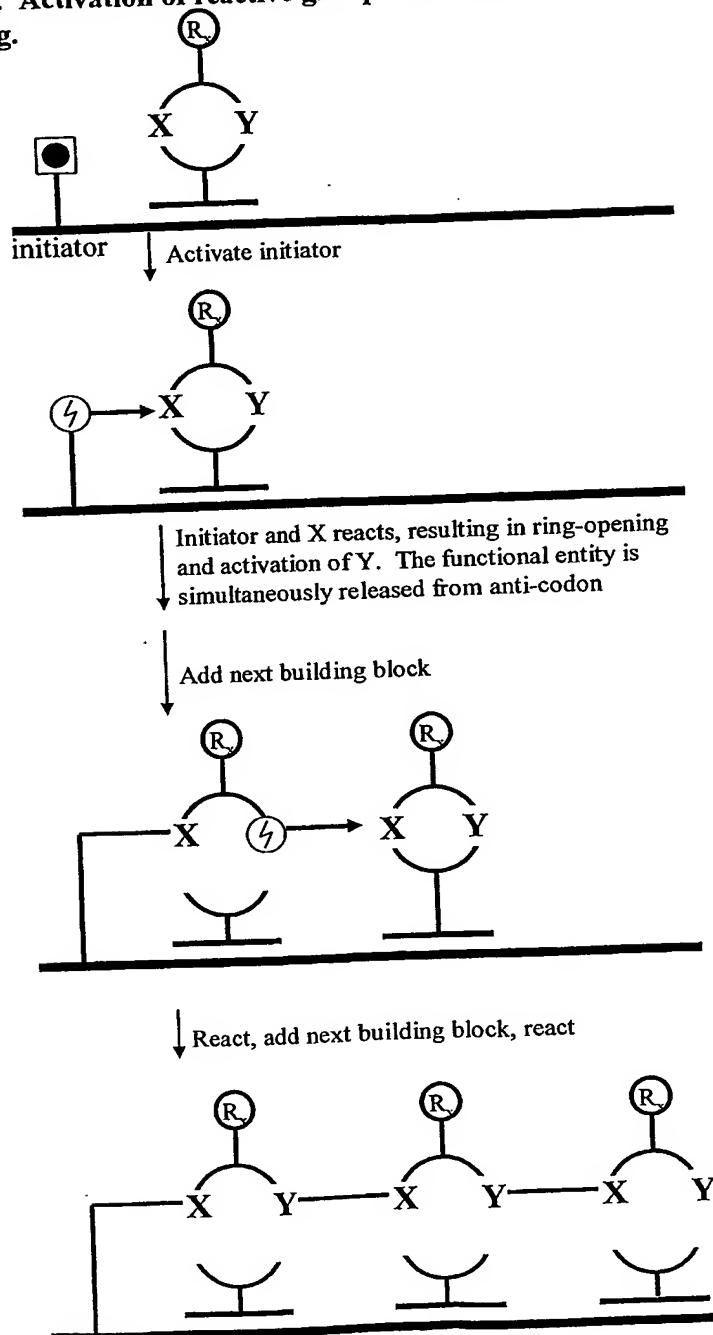
Fig. 11. Templated synthesis of a polymer, using non-simultaneous reaction and cleavage.



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Fig. 12. Activation of reactive group and release from anti-codon by ring opening.



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Fig. 13. Symmetric fill-in reaction (symmetric XX building blocks).

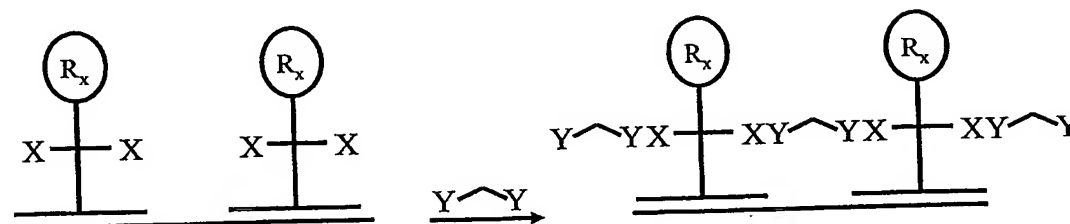
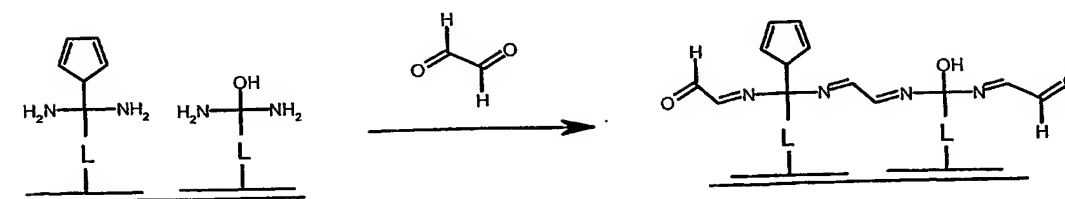
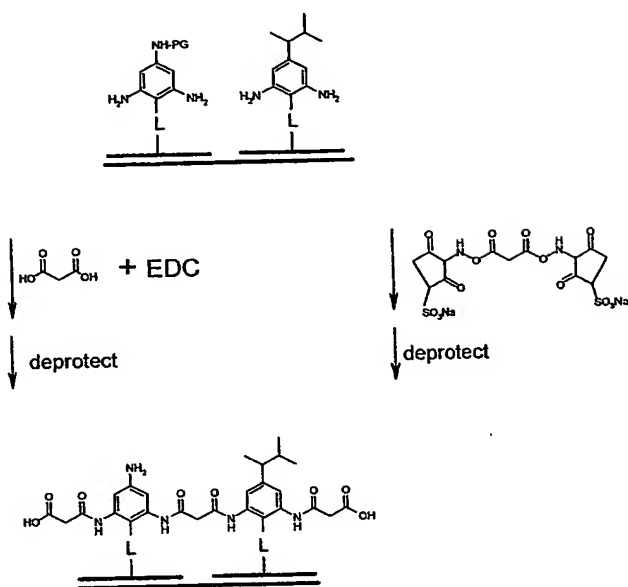
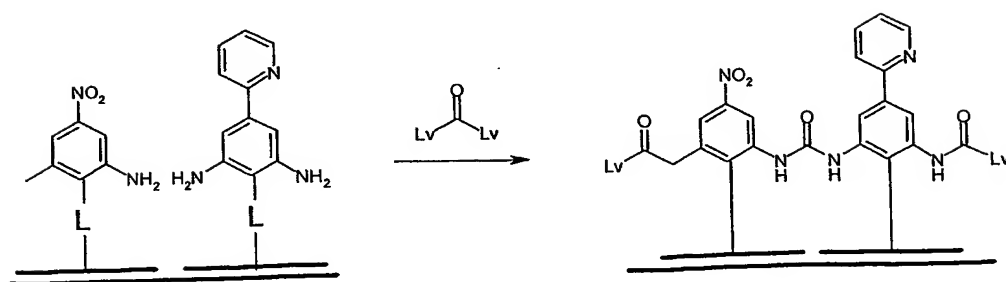


Fig. 13, ex 1. Imine formation by fill-in.



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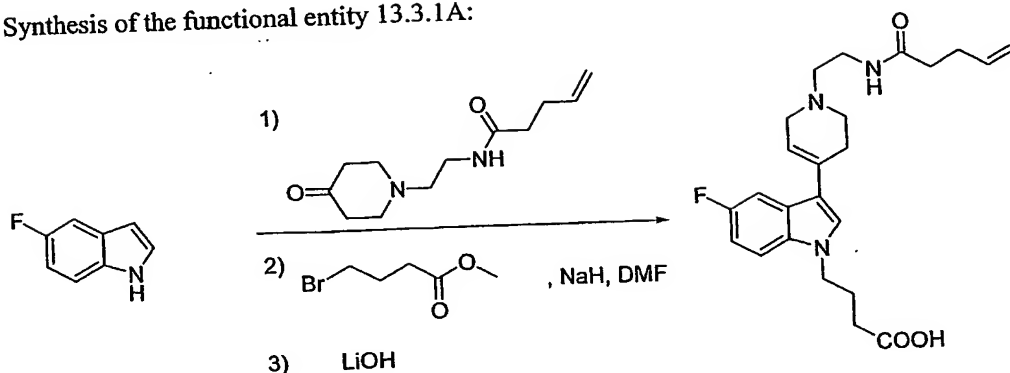
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Fig. 13, example 2. Amide formation.**Fig. 13, example 3. Urea formation**

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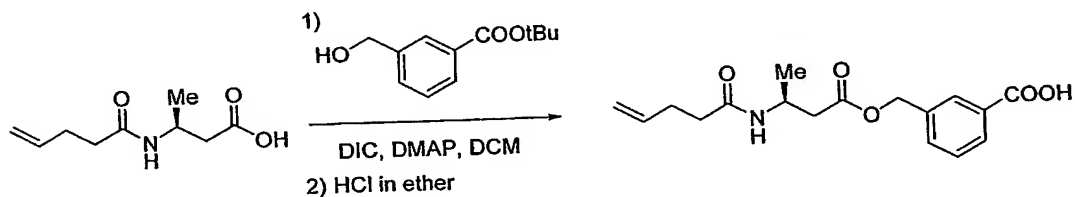
Fig 13, ex 3.1 Urea formation

Synthesis of the functional entity 13.3.1A:



5-Fluoroindole (1 eq) is dissolved in ethanol and treated with pent-4-enoic acid [2-(4-oxo-piperidin-1-yl)-ethyl]-amide (1.2 eq) and 2N KOH. The mixture is stirred o/n at reflux. The crude is evaporated and purified by silica gel filtration. The purified material is treated with methyl 3-bromobutanoate (1.2 eq) and NaH (1.5 eq) in DMF at rt. After 5 hours LiOH (10 eq) and water is added and the reaction mixture is stirred at rt o/n. The final product is purified by LC-MS and loaded on a DNA oligo containing an amino function.

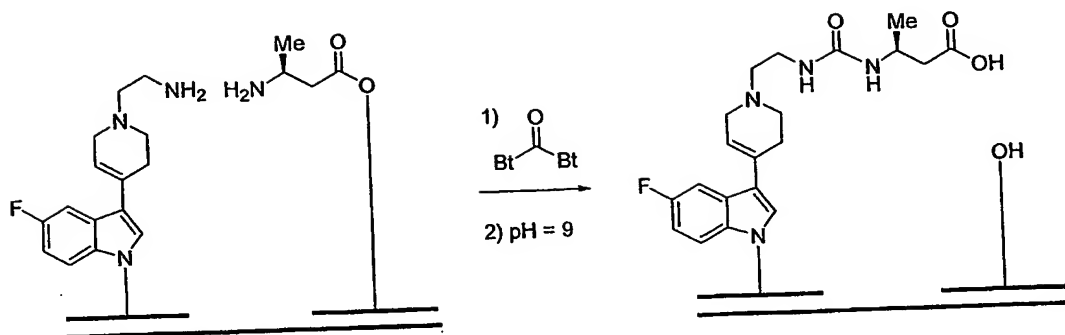
Synthesis of the functional entity 13.3.1B:



3-Pent-4-enoylamino-butanoic acid (1 eq) is treated with 3-hydroxymethyl-benzoic acid tert-butyl ester (1.2 eq), DIC (1.2 eq) and DMAP (0.2 eq) in DCM. The reaction mixture is stirred o/n at rt. The crude is evaporated and purified by silica gel filtration. The purified material is dissolved in diethyl ether and treated with HCl in diethyl ether. After stirring for 3 hours the mixture is evaporated and the crude material loaded on a DNA oligo containing an amino function.

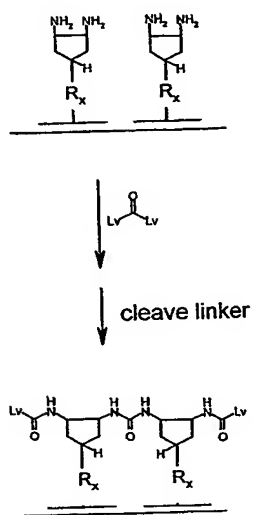
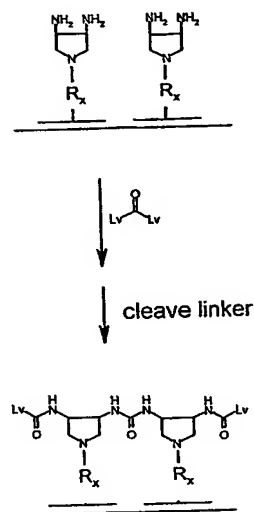
Fill in experiment using functional entity 13.3.1A and 13.3.1B:

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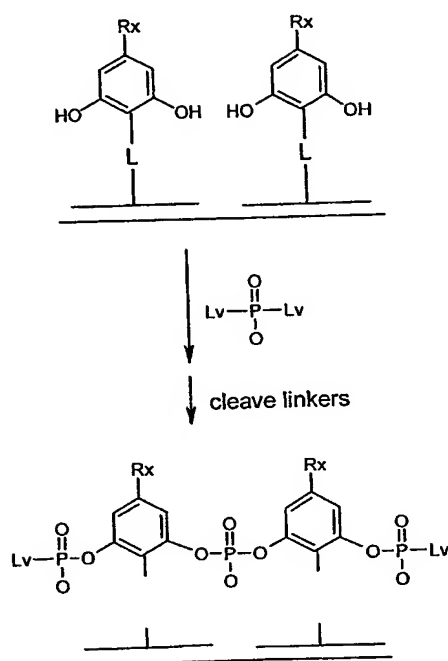
The two loaded oligos are mixed with a template oligo in in hepes buffer (pH = 7.5) and 100 mM NaCl. 1,1'-Carbonylbisbenzotriazole (0.1M in MeOH) is added and the mixture is left at rt for 4 hours. pH is then adjusted to 9 and the mixture is left at rt o/n.

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Fig. 13, ex 4. Chiral and non-chiral templated molecule**A.****B.**

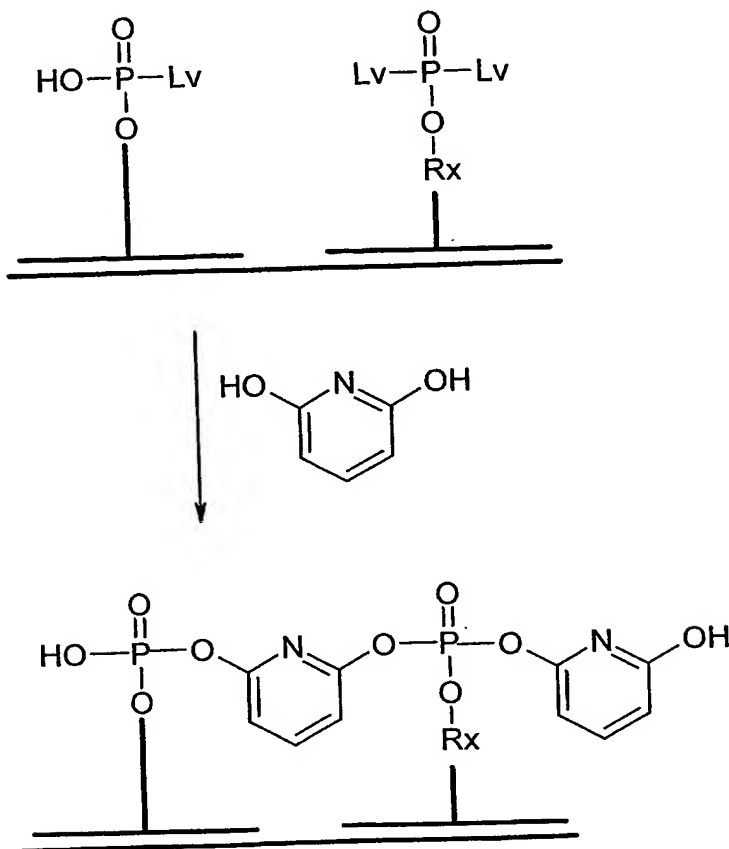
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Fig. 13, ex 5. Symmetric fill-in: Formation of a phosphodiester bond.



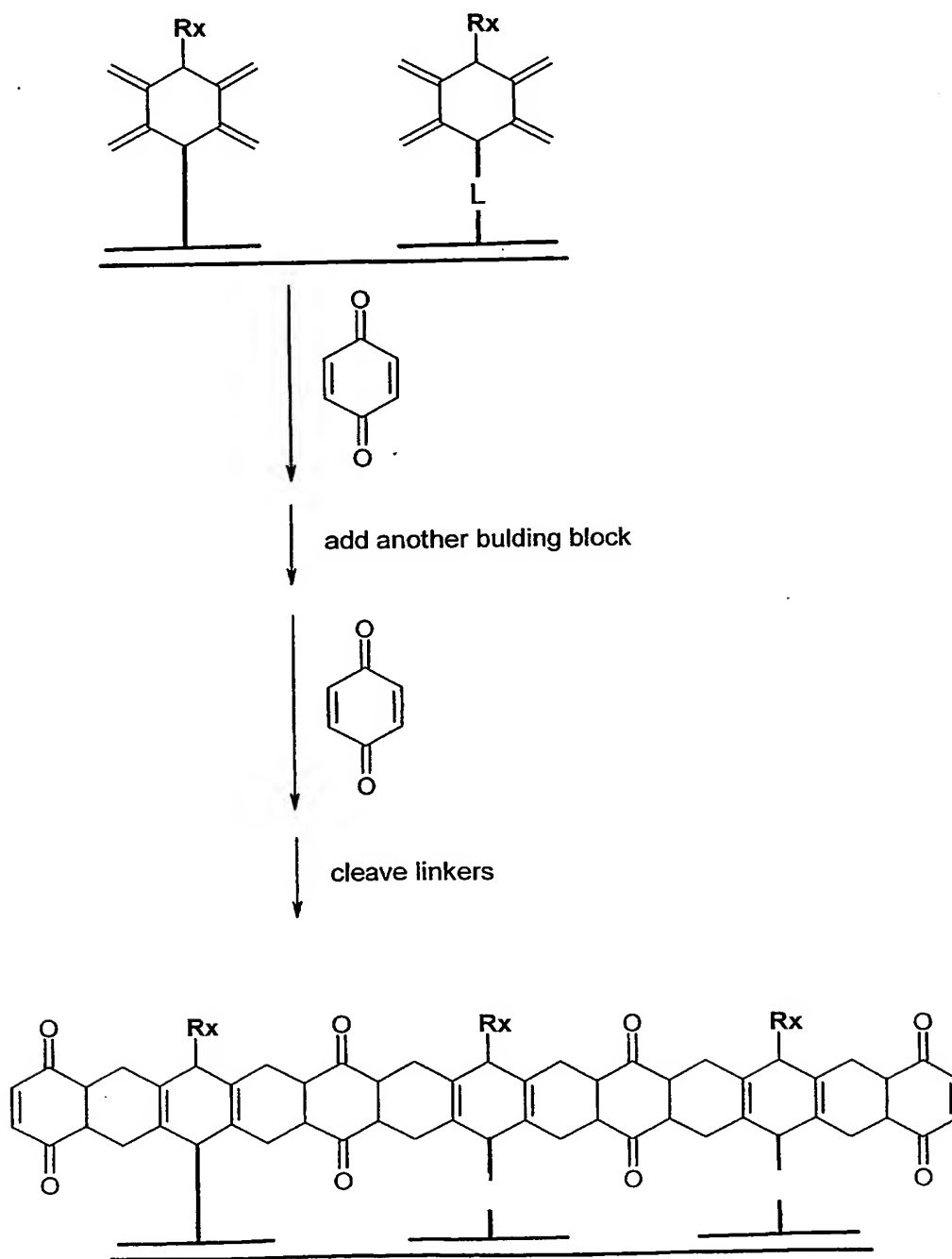
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Fig. 13, ex 6. Fill-in: Phosphodiester formation with one reactive group in each building block



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Fig. 13, ex 7. Pericyclic reaction.

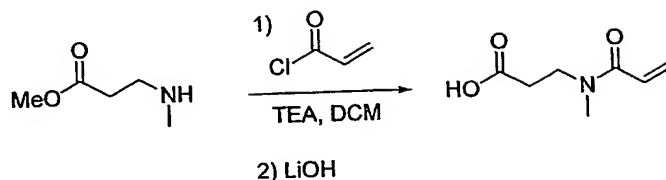


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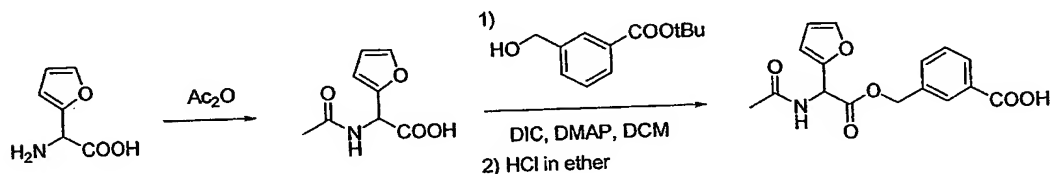
Figure 13, ex 7.1 Pericyclic reaction

Synthesis of the functional entity 13.7.1A:



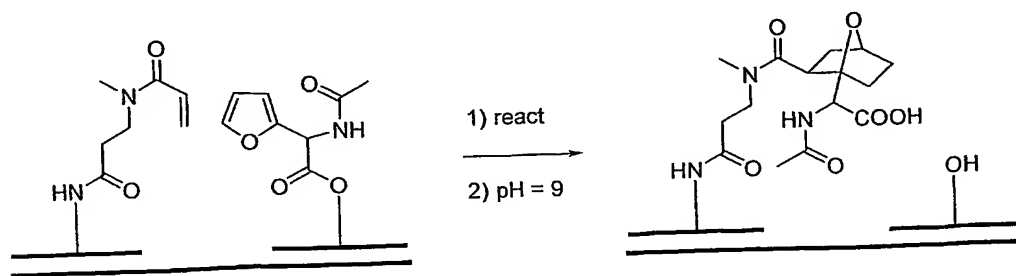
3-Methylamino-propionic acid methyl ester (1eq) is dissolved in DCM and triethylamine (2 eq). The mixture is cooled to 0° and treated with acryloyl chloride (1.5 eq). After 2 hours the reaction mixture is evaporated, redissolved in THF and treated with LiOH (10 eq) and water. The mixture is left at rt for 3 hours. The crude is extracted with EtOAc (2x). The combined organic phases are dried over MgSO₄ and evaporated. The product is purified by LC-MS and loaded on a DNA oligo containing an amino function.

Synthesis of the functional entity 13.7.1B:



Amino-furan-2-yl-acetic acid (1eq) is treated with acetic anhydride (3 eq) at rt for 1 hour. The crude is evaporated and the product purified by LC-MS and then treated with 3-hydroxymethyl-benzoic acid tert-butyl ester (1.2 eq), DIC (1.2 eq) and DMAP (0.2 eq) in DCM. The reaction mixture is stirred o/n at rt. The crude is evaporated and purified by silica gel filtration. The purified material is dissolved in diethyl ether and treated with HCl in diethyl ether. After stirring for 3 hours the mixture is evaporated and the crude material loaded on a DNA oligo containing an amino function.

Pericyclic reaction experiment using functional entity 13.7.1A and 13.7.1B:

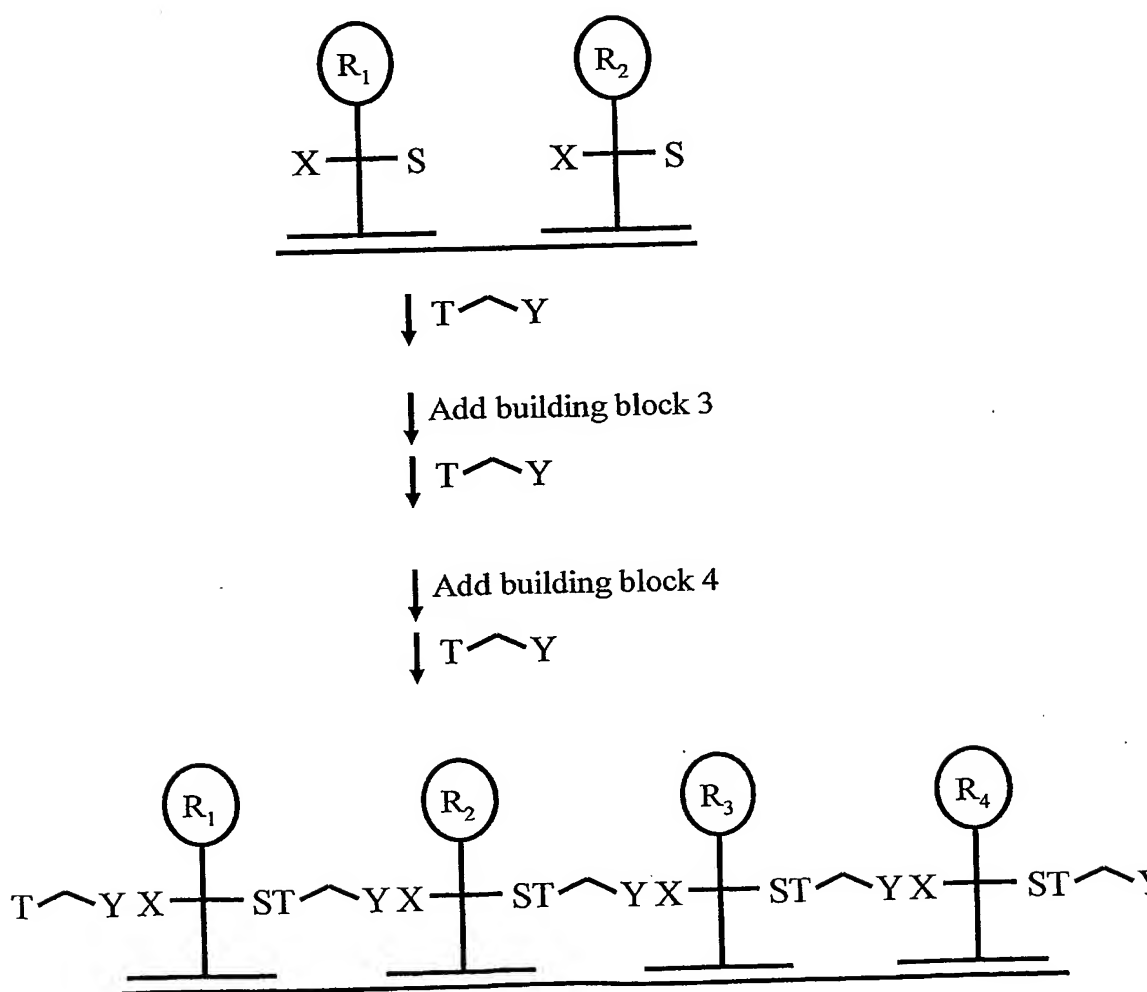


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The two loaded oligos are mixed with a template oligo in in hepes buffer (pH = 7.5) and 100 mM. The mixture is left at rt for 4 hours. pH is then adjusted to 9 and the mixture is left at rt o/n.

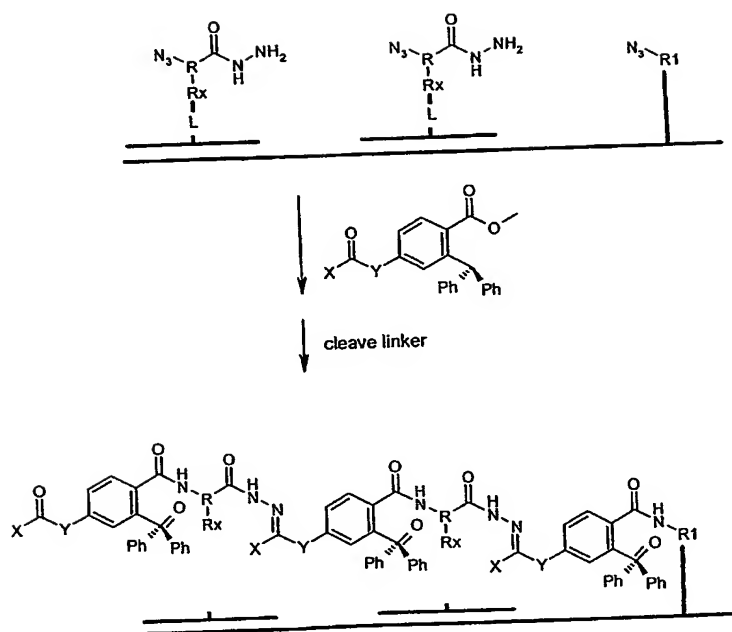
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Fig. 13. "Fill-in" reaction (asymmetric XS monomers).

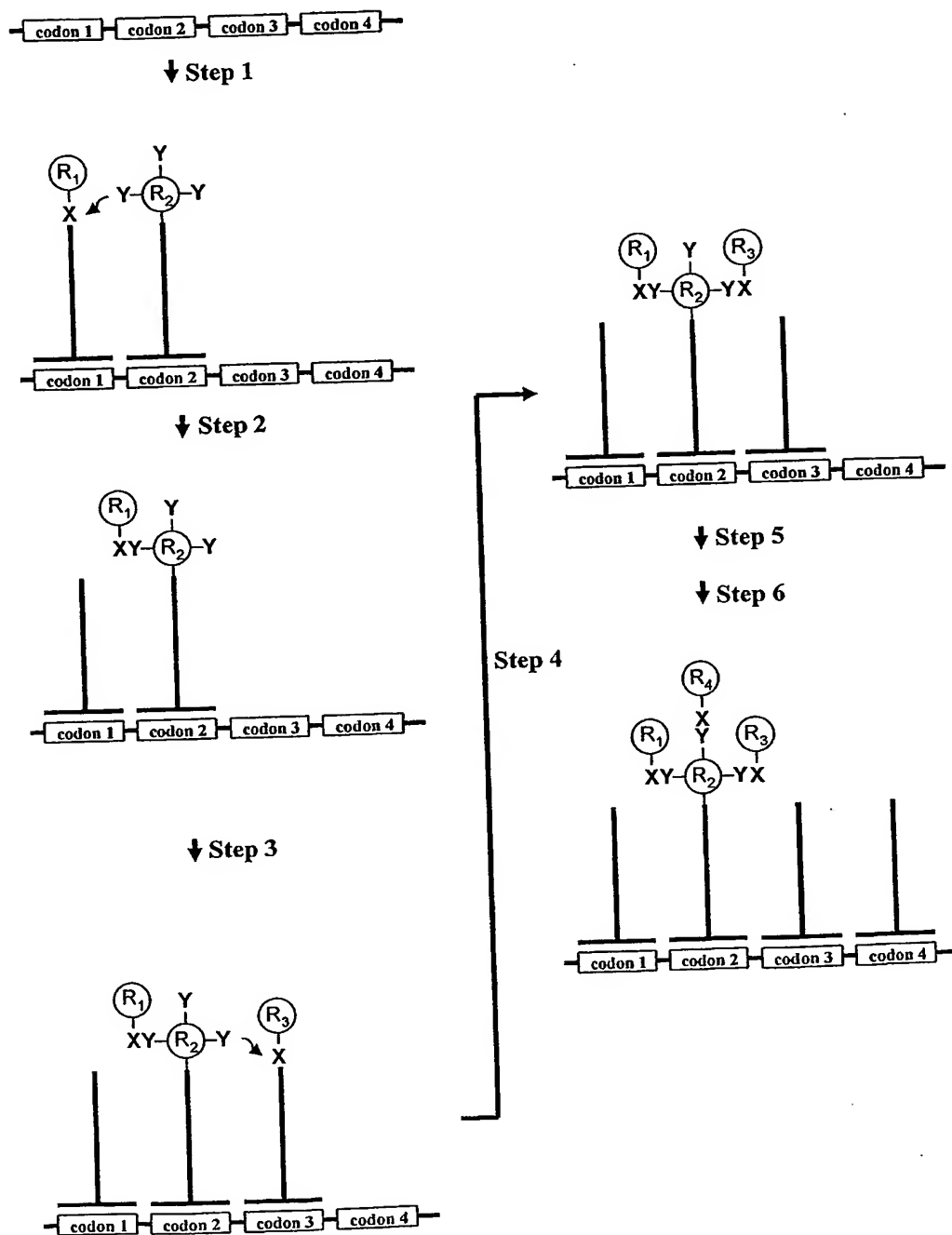
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Fig. 14, example 1.



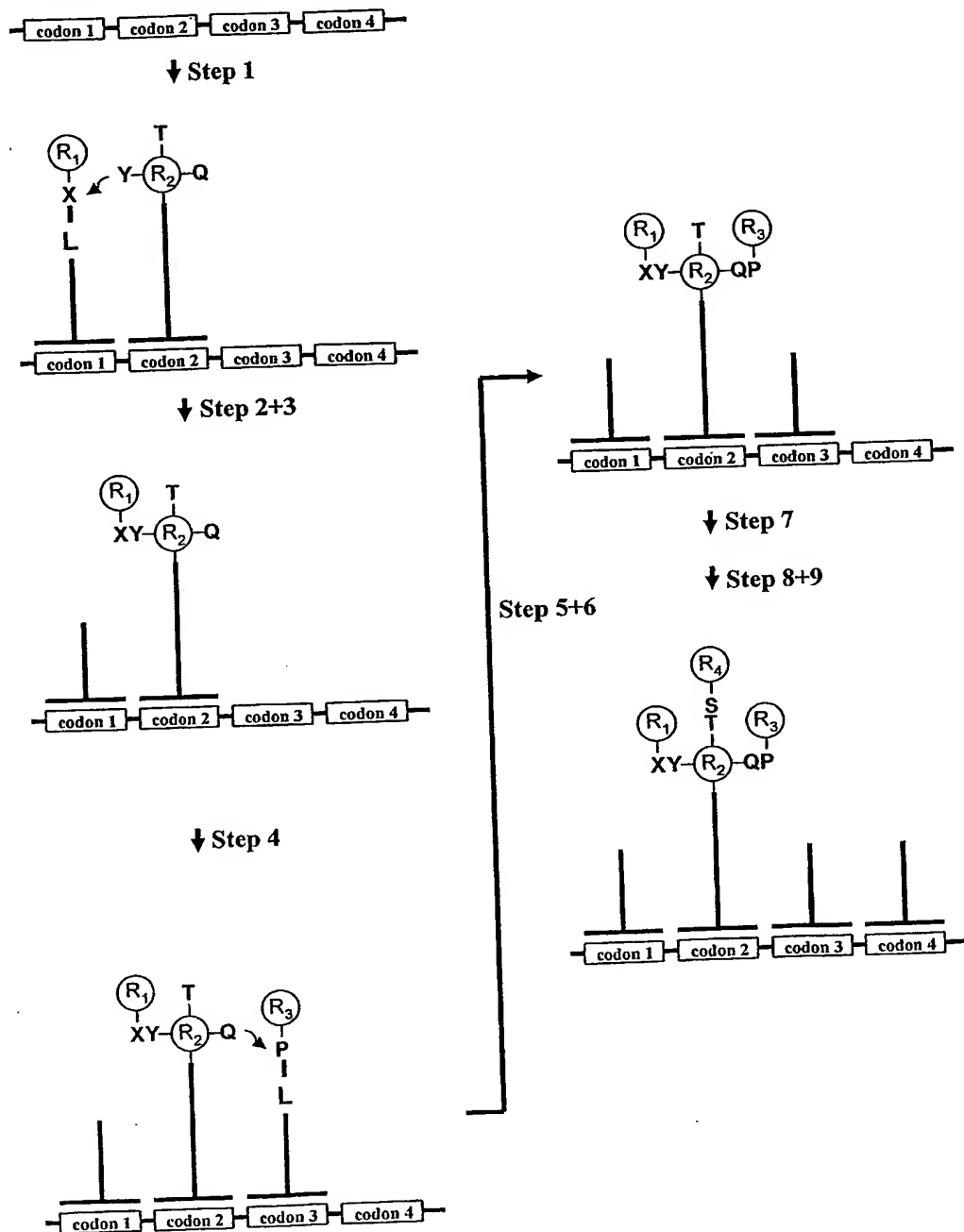
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Fig. 15. Templated synthesis of a non-linear molecule.

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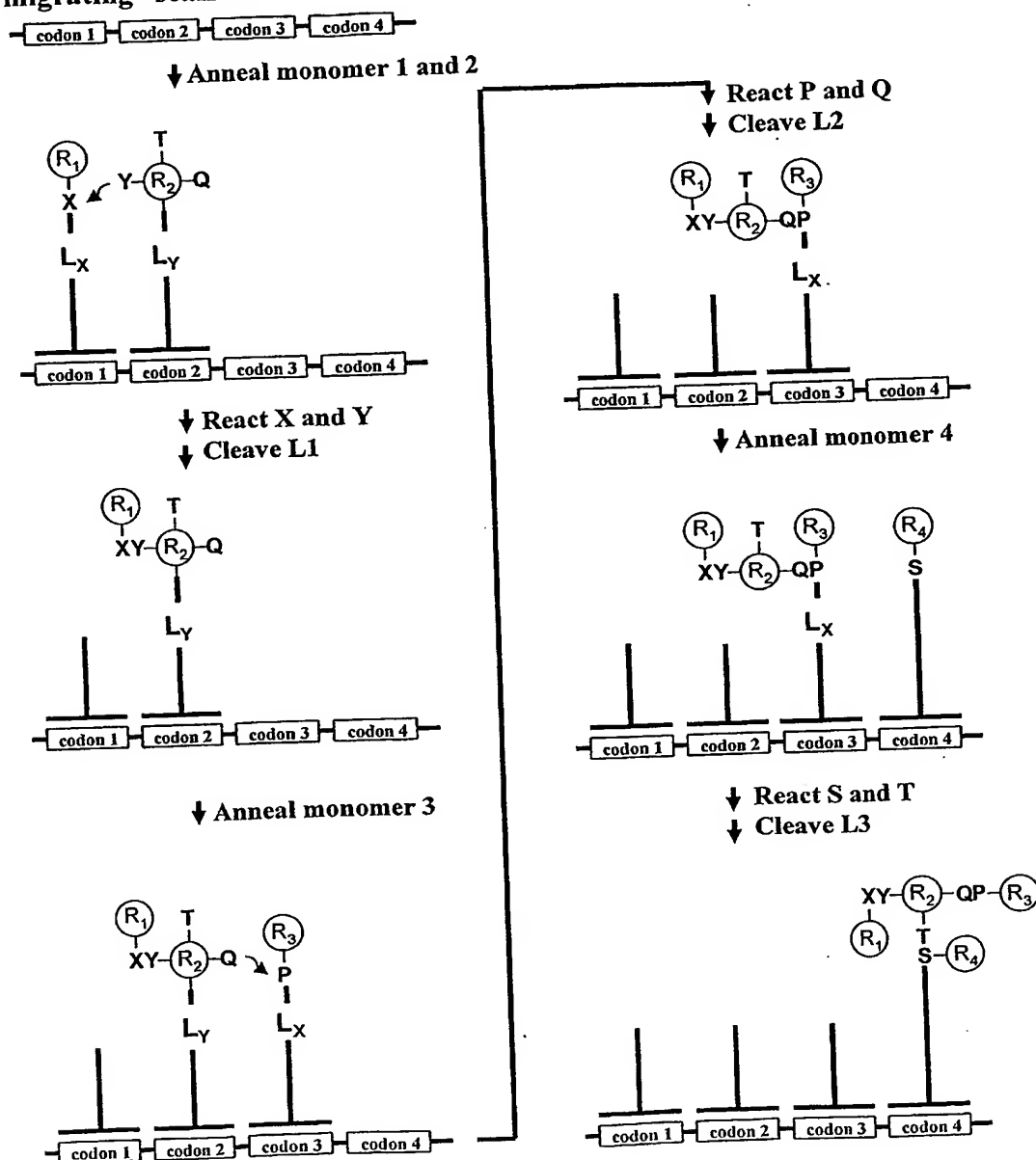
Fig. 16. Templated synthesis of a non-linear molecule, employing reactive groups of different classes, and non-simultaneous reaction and cleavage.



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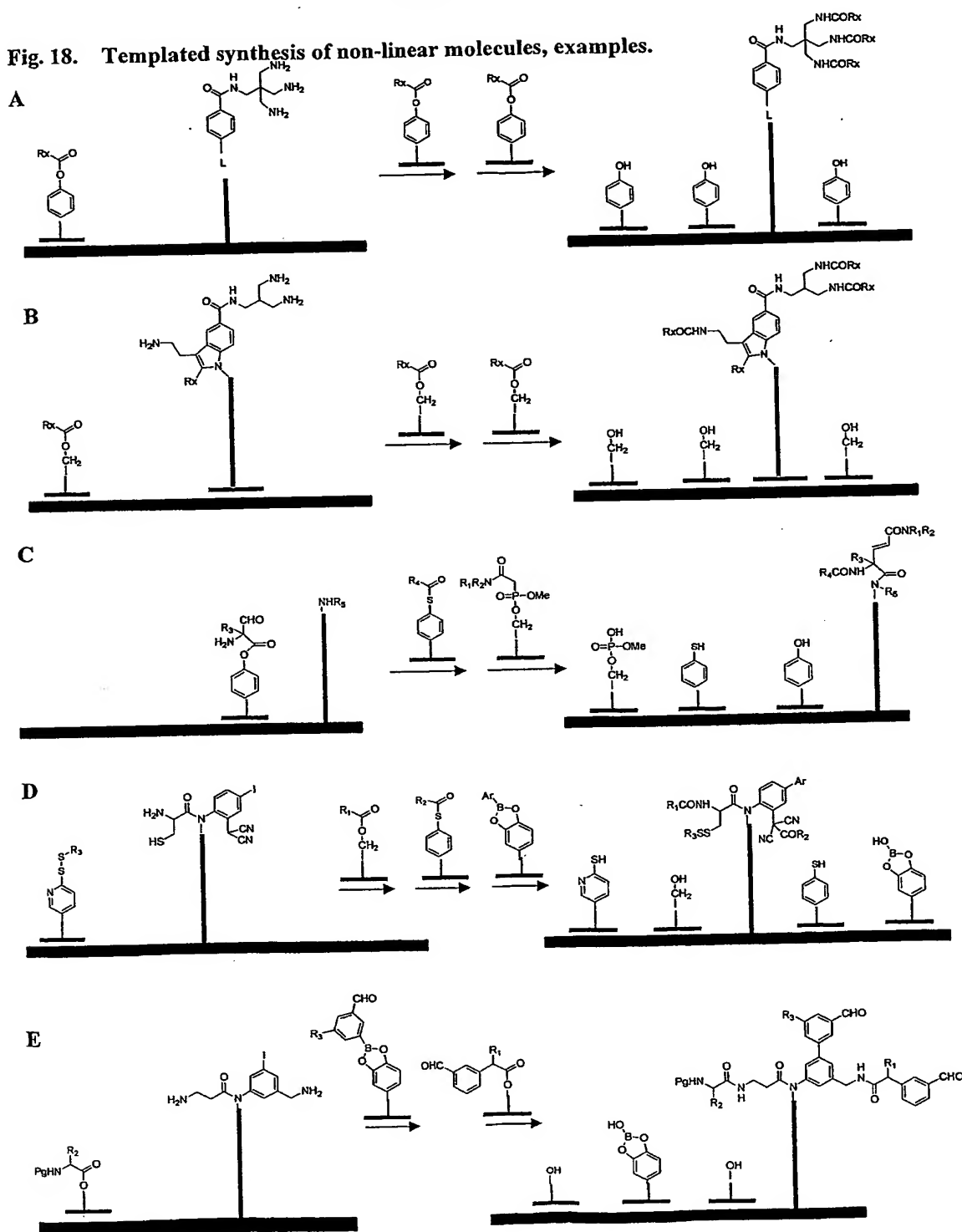
Fig. 17. Migrating scaffold. Templated synthesis of a non-linear molecule, by exploiting the increased proximity effect that arises from a "migrating" scaffold.



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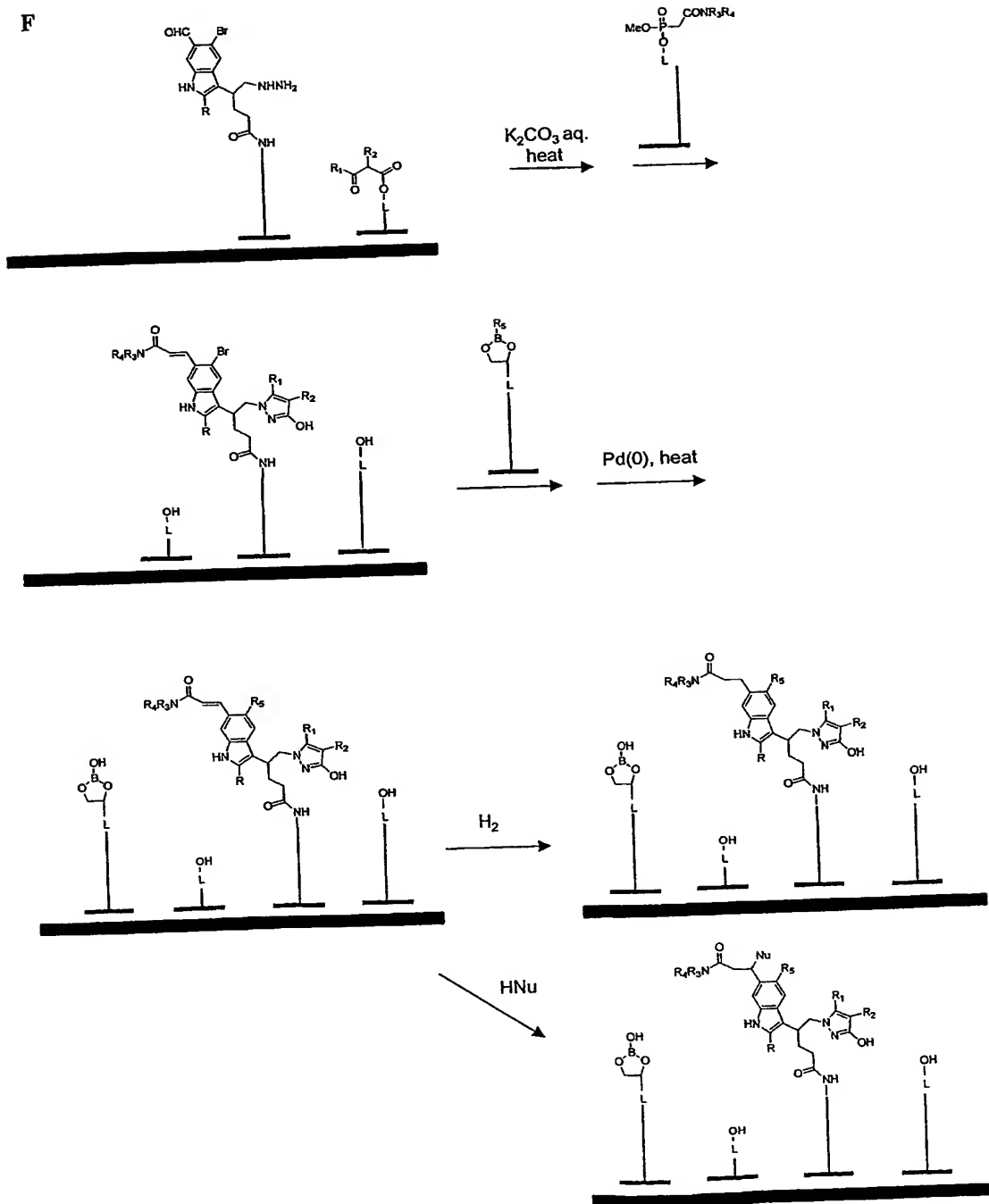
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Fig. 18. Templated synthesis of non-linear molecules, examples.



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Fig. 19. Templatd synthesis in whiche the reaction step is performed under conditions where specific annealing of building block to template is inefficient.

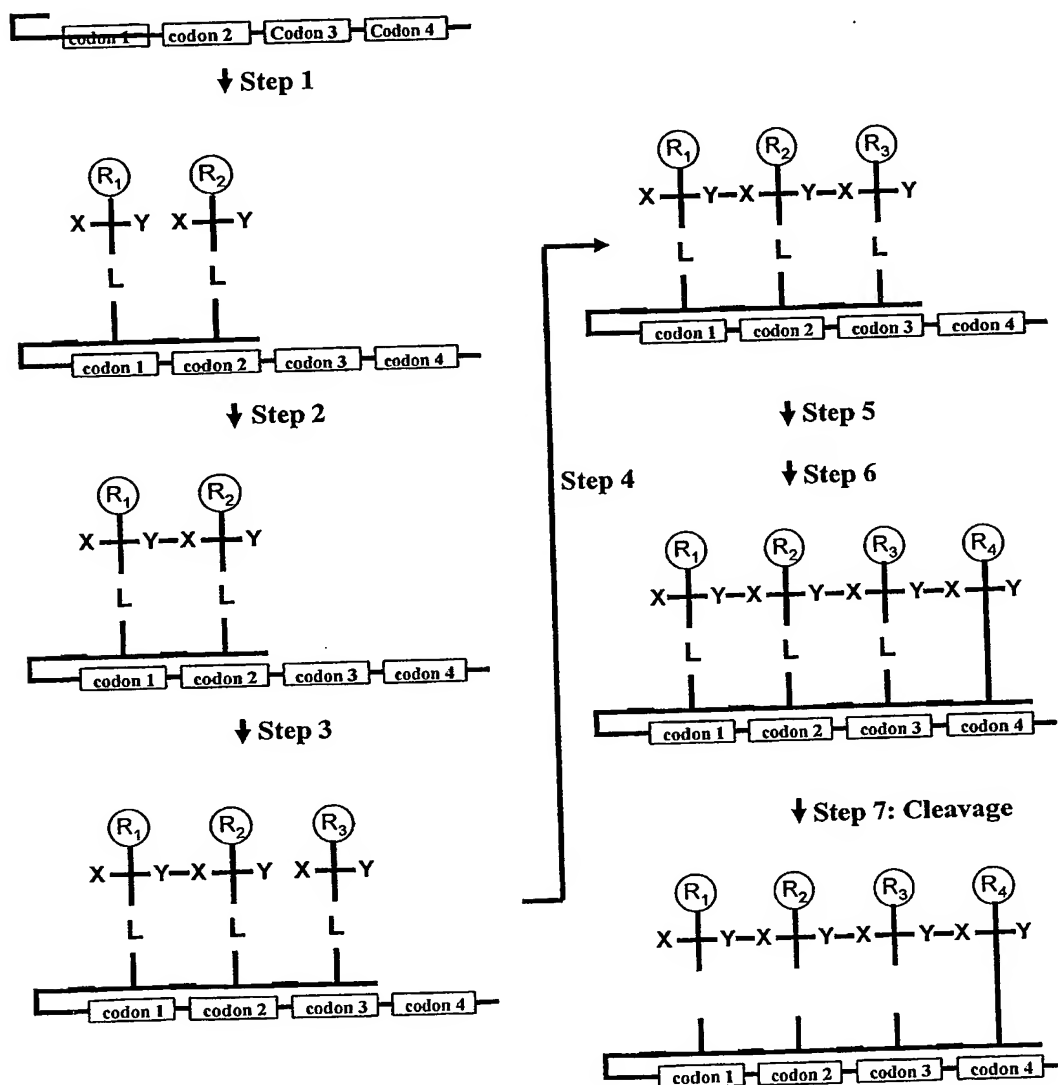
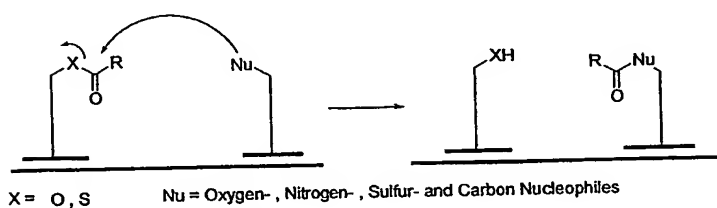
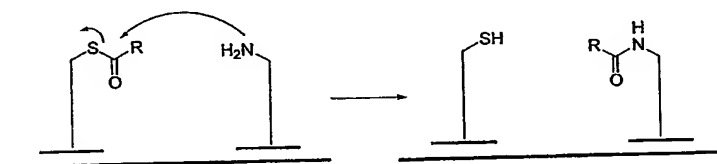
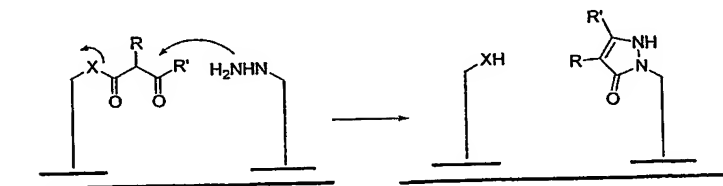
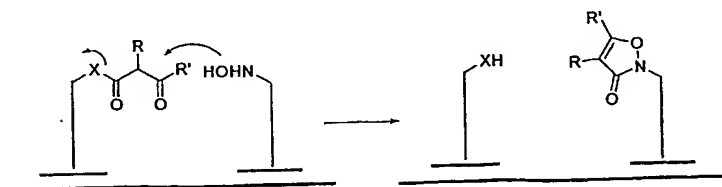
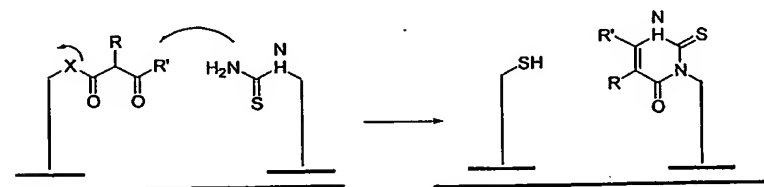
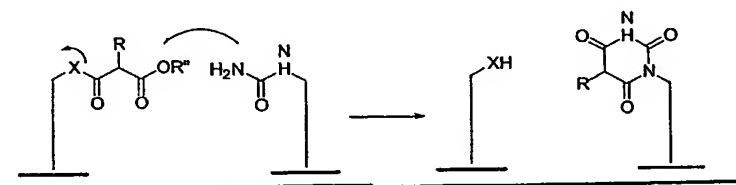
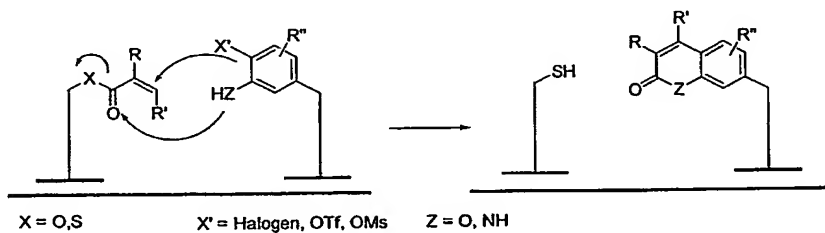
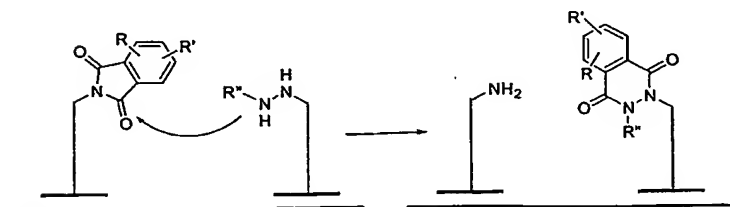
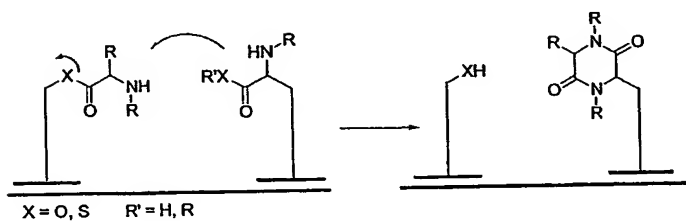
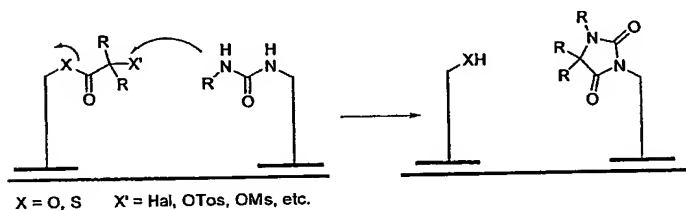
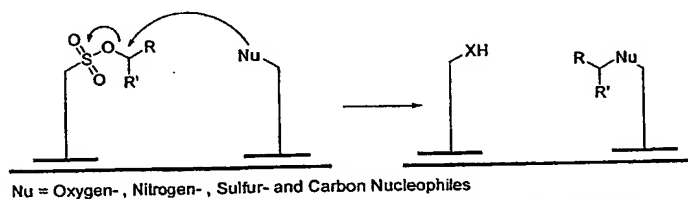
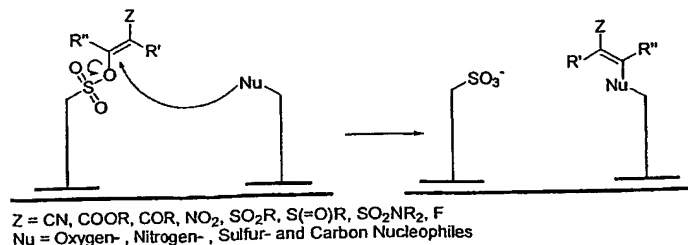


Fig. 20. Reaction types allowing simultaneous reaction and activation.**Nucleophilic substitution using activation of electrophiles****A. Acylating monomer building blocks - principle****B. Acylation****Amide formation by reaction of amines with activated esters****C. Acylation****Pyrazolone formation by reaction of hydrazines with β -Ketoesters****D. Acylation****Isoxazolone formation by reaction of hydroxylamines with β -Ketoesters**

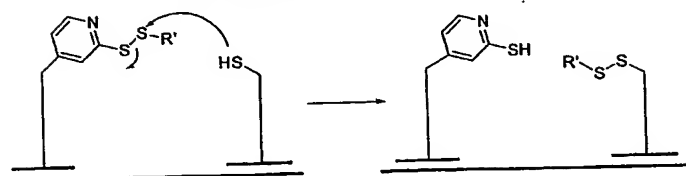
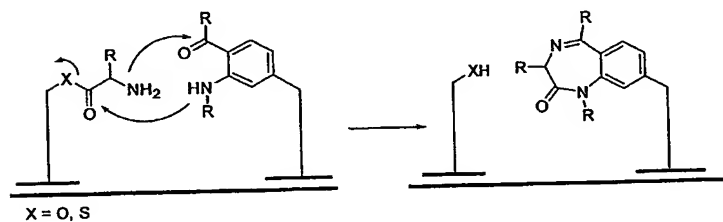
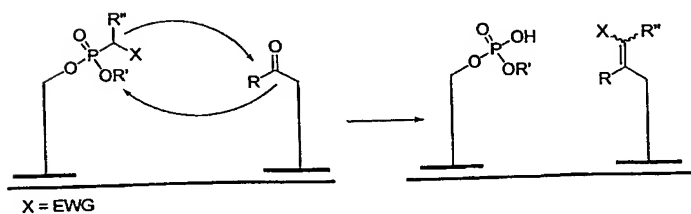
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E. Acylation**Pyrimidine formation by reaction of thioureas with β -Ketoesters****F. Acylation****Pyrimidine formation by reaction of ureas with Malonates****G. Acylation****Coumarine or quinolinon formation by a Heck reaction followed by a nucleophilic substitution****H. Acylation****Phthalhydrazide formation by reaction of Hydrazines and Phthalimides****SUBSTITUTE SHEET (RULE 26)**

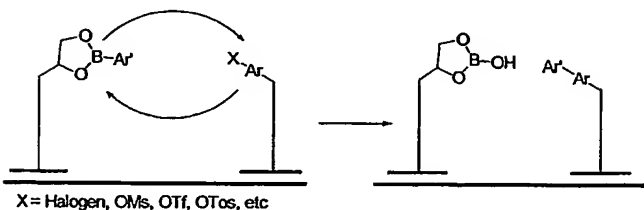
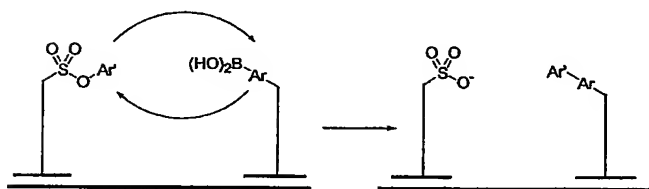
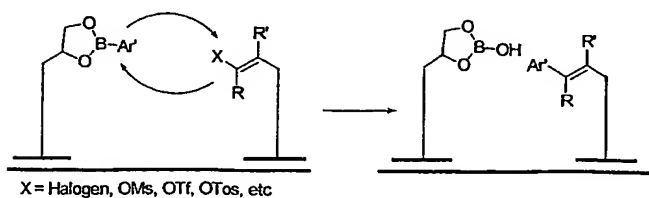
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I. Acylation**Diketopiperazine formation by reaction of Amino Acid Esters****J. Acylation****Hydantoin formation by reaction of Urea and α -substituted Esters****K. Alkylating monomer building blocks - principle****Alkylated compounds by reaction of Sulfonates with Nucleophiles****L. Vinylating monomer building blocks - principle**

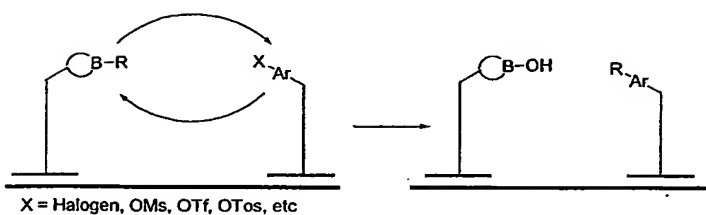
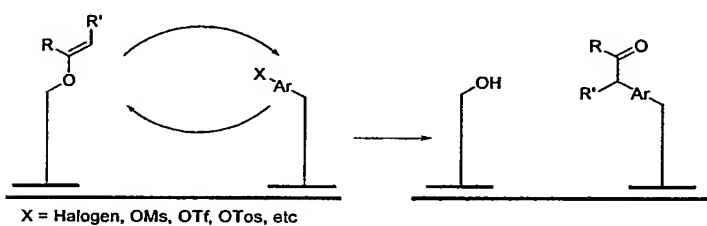
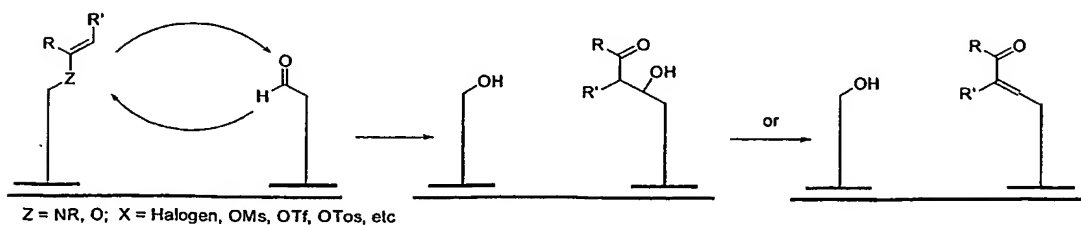
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M. Heteroatom electrophiles**Disulfide formation by reaction of Pyridyl disulfide with Mercaptanes****N. Acylation****Benzodiazepinone formation by reaction of Amino Acid Esters and Amino Ketones****Addition to carbon-hetero multiple bonds****O. Wittig/Horner-Wittig-Emmons reagents****Substituted alkene formation by reaction of Phosphonates with Aldehydes or Ketones**

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Transition metal catalysed reactions**P. Arylation****Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls****Q. Arylation****Biaryl formation by the reaction of Boronates with Aryls or Heteroaryls****R. Arylation****Vinylarene formation by the reaction of alkenes with Aryls or Heteroaryls**

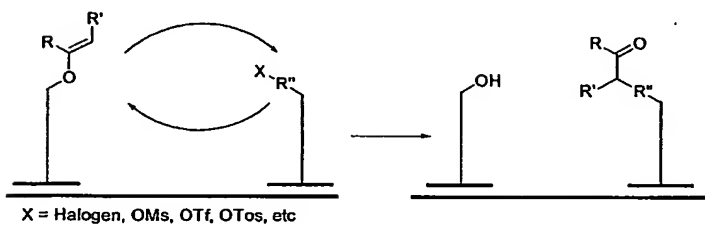
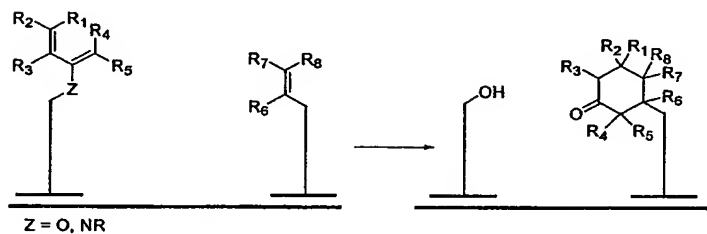
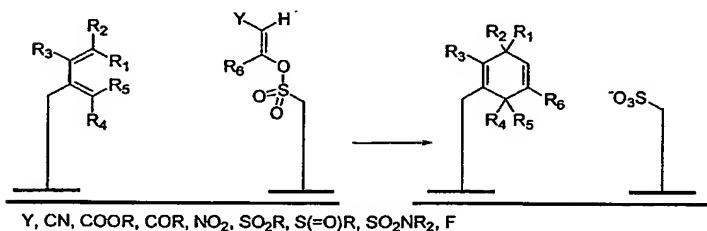
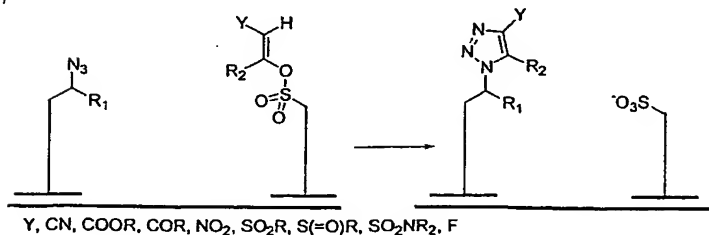
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S. Alkylation**Alkylation of arenes/hetarens by the reaction with Alkyl boronates****T. Alkylation****Alkylation of arenes/hetarens by reaction with enolethers****Nucleophilic substitution using activation of nucleophiles****U. Condensations****Alkylation of aldehydes with enolethers or enamines****SUBSTITUTE SHEET (RULE 26)**

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V. Alkylation

Alkylation of aliphatic halides or tosylates with enolethers or enamines

**Cycloadditions****W. [2+4] Cycloadditions****X. [2+4] Cycloadditions****Y. [3+2] Cycloadditions**

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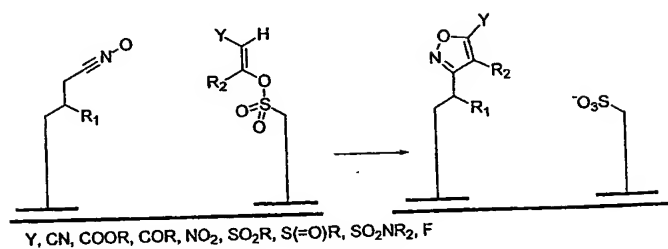
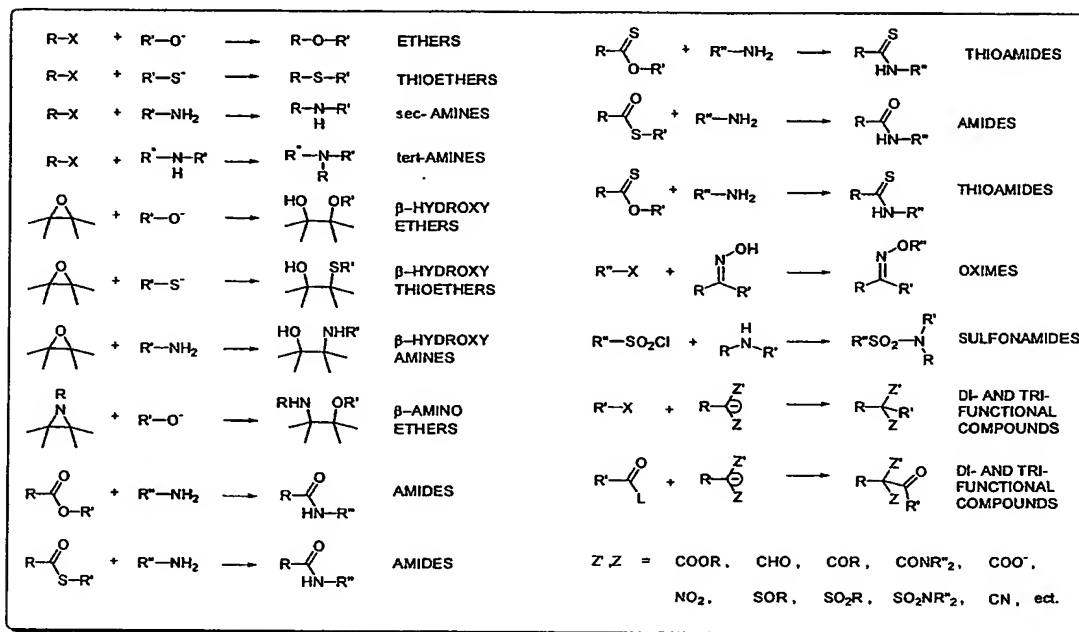
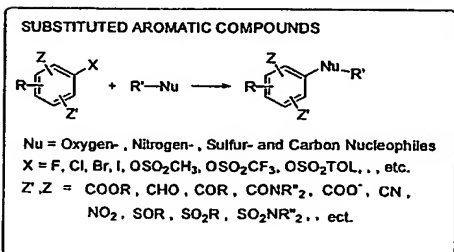
Z. [3+2] Cycloadditions

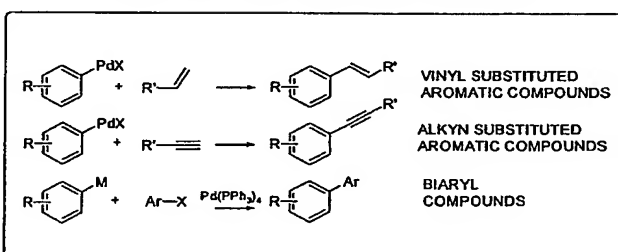
Fig. 21. Pairs of reactive groups X,Y and the resulting bond XY.
Nucleophilic substitution reaction



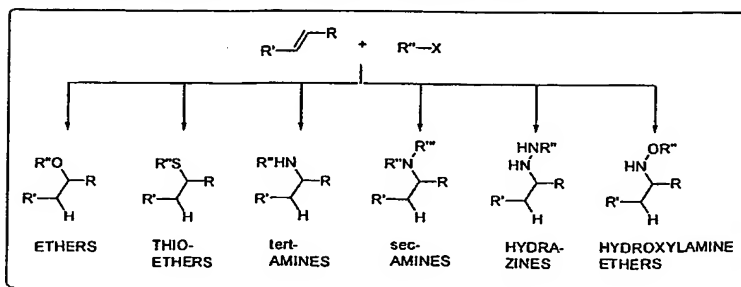
Aromatic nucleophilic substitution



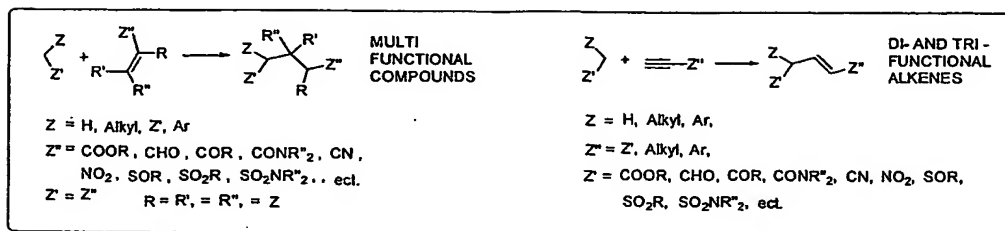
Transition metal catalysed reactions



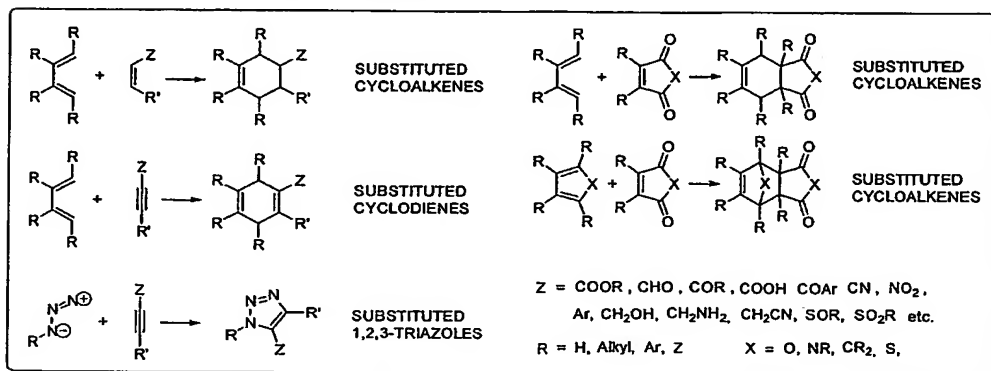
Addition to carbon-carbon multiplebonds



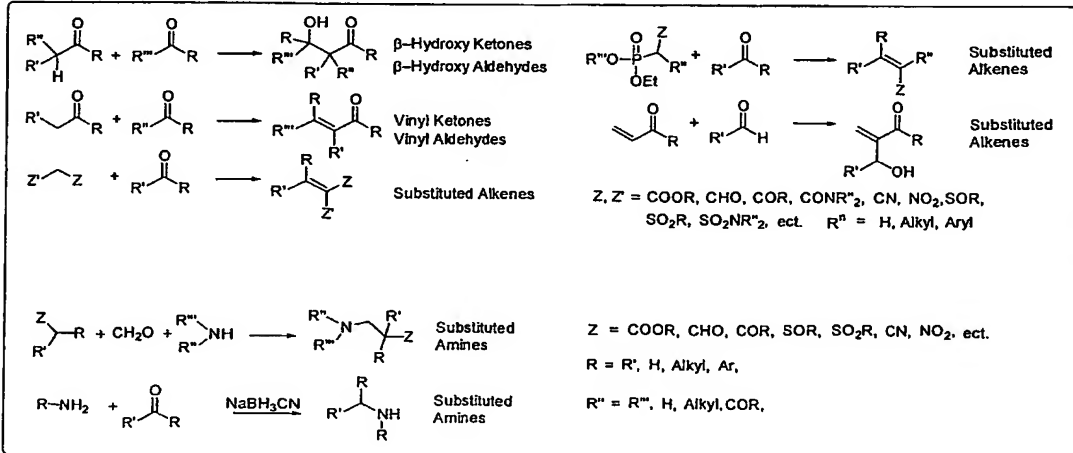
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Cycloaddition to multiple bonds

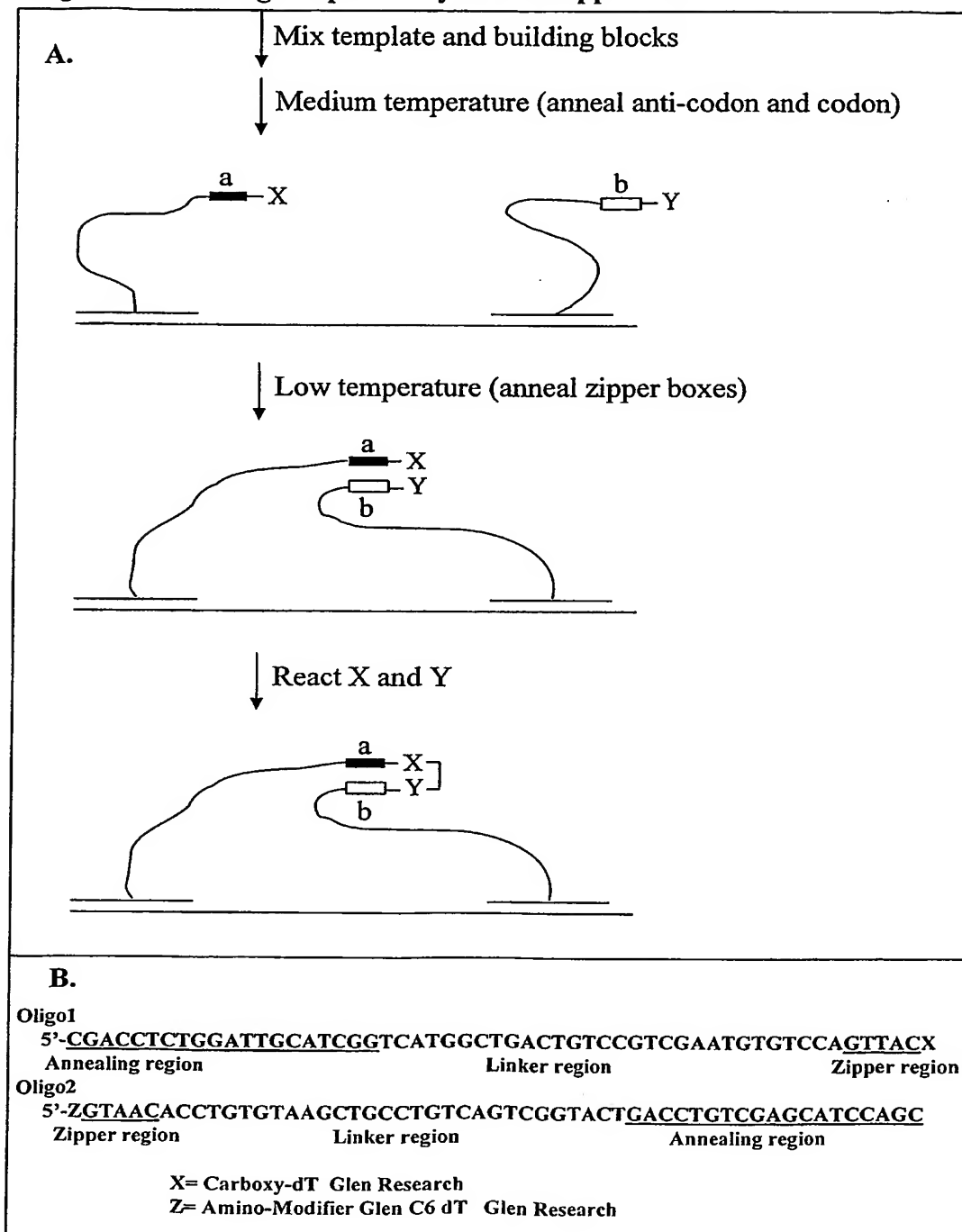


Addition to carbon-hetero multiple bonds



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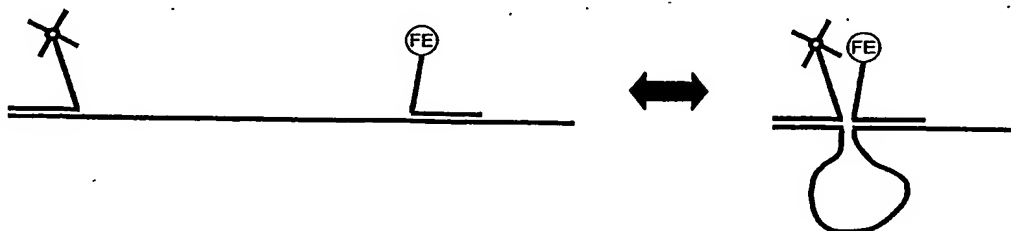
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Fig. 22. Increasing the proximity effect: Zipper box.

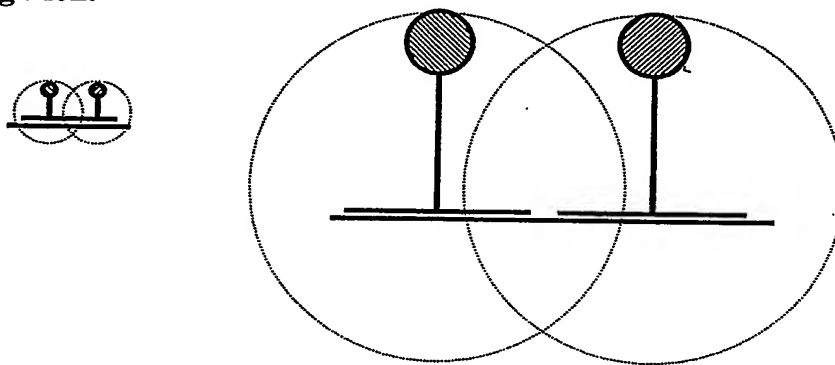
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Fig. 23. Increasing the proximity effect: Helix Stacking (A), Ligation (B), and (C) Rigid linkers

A. Double helix stacking.



B. Ligation.



C. Rigid linkers.

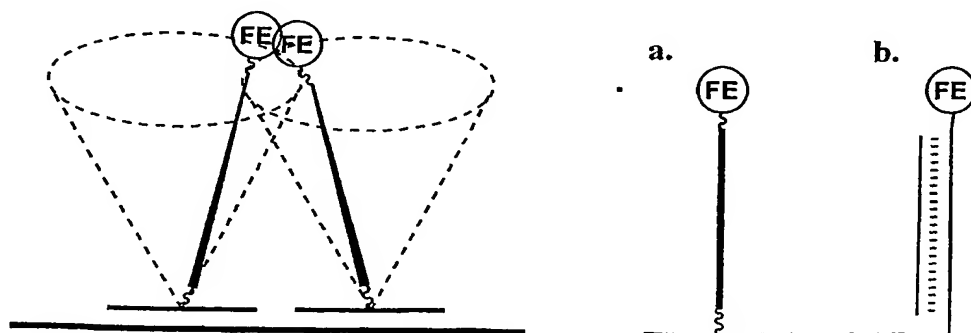
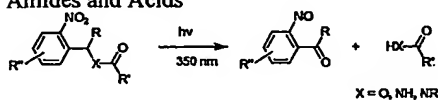
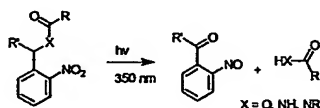


Fig. 24. Cleavable Linkers

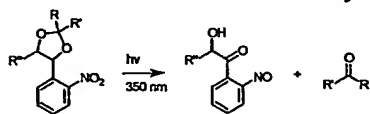
A. Linker for the formation of Ketones, Aldehydes, Amides and Acids



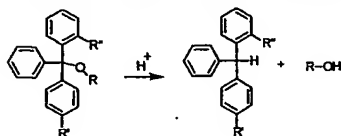
B. Linker for the formation of Ketones, Amides and Acids



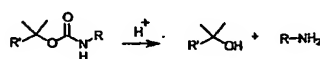
C. Linker for the formation of Aldehydes and Ketones



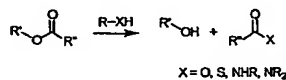
D. Linker for the formation of Alcohols and Acids



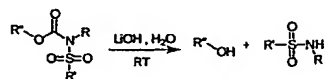
E. Linker for the formation of Amines and Alcohols



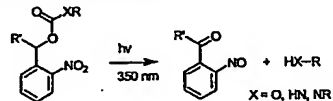
F. Linker for the formation of Esters, Thioesters, Amides and Alcohols



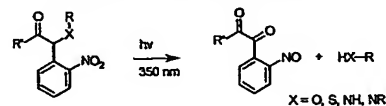
G. Linker for the formation of Sulfonamides and Alcohols



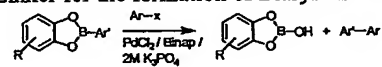
H. Linker for the formation of Ketones, Amines and Alcohols



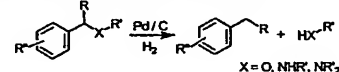
I. Linker for the formation of Ketones, Amines, Alcohols and Mercaptanes



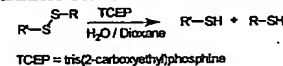
J. Linker for the formation of Biaryl and Bihetaryl



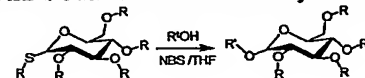
K. Linker for the formation of Benzyles, Amines, Anilins Alcohols and Phenoles



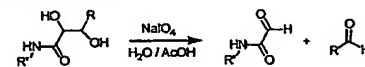
L. Linker for the formation of Mercaptanes



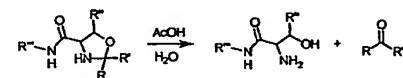
M. Linker for the formation of Glycosides



N. Linker for the formation of Aldehydes and Glyoxylamides

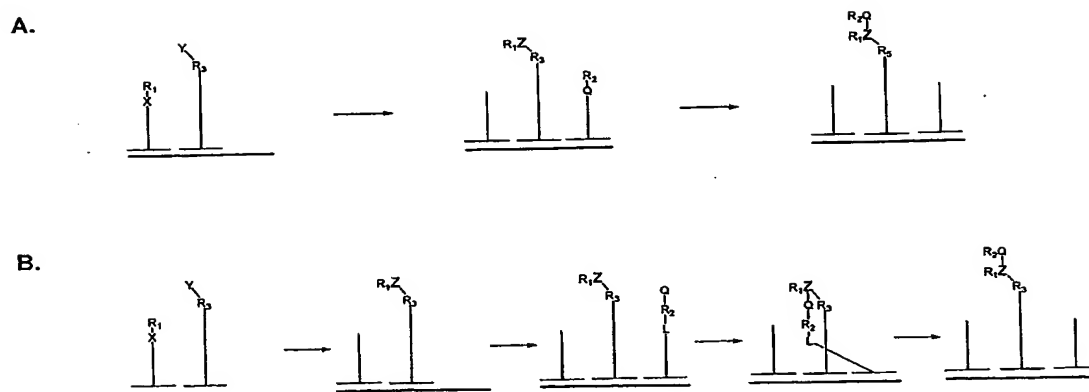


O. Linker for the formation of Aldehydes, Ketones And Aminoalcohols



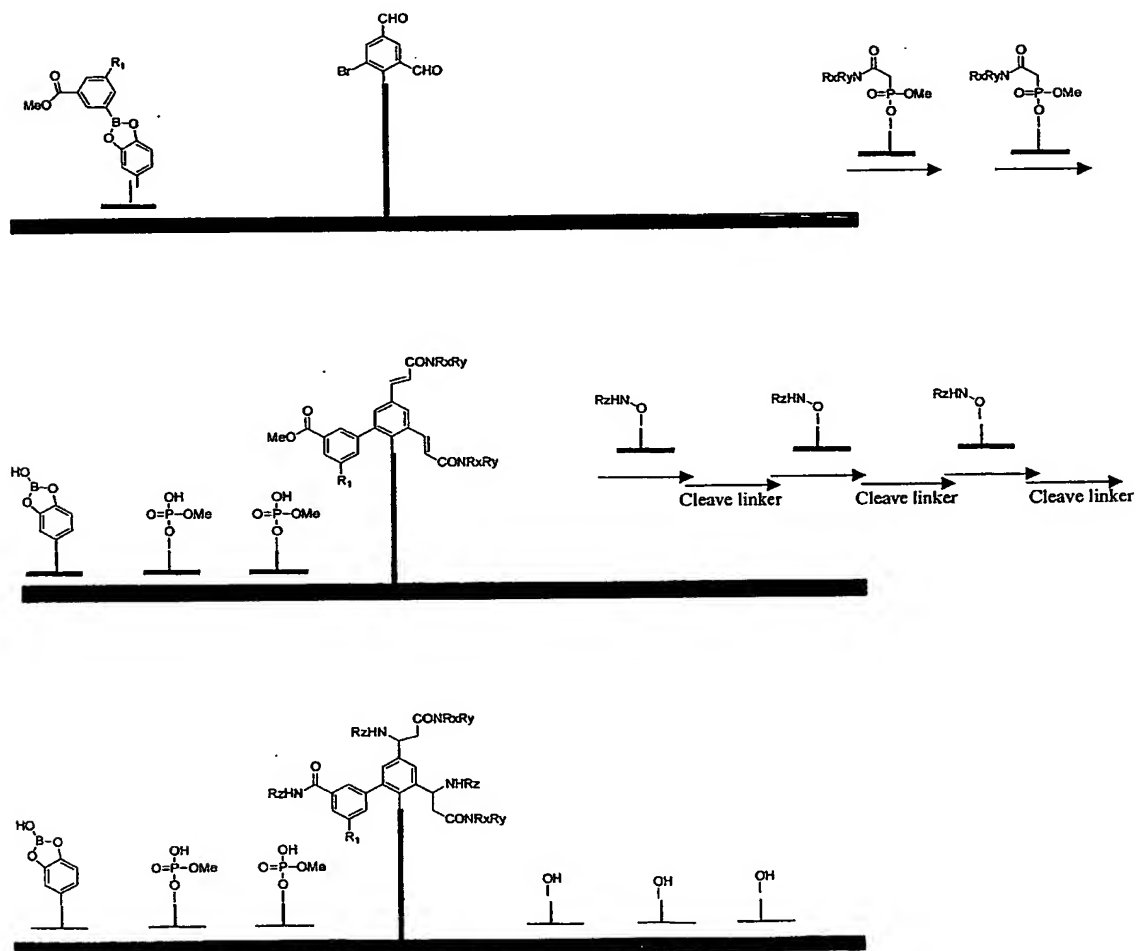
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Fig. 25. Templated synthesis by generating a new reactive group.



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Fig. 25, example 1. Generation of reactive groups in the first reaction round, followed by reaction of the generated reactive groups with introduced reactive groups.



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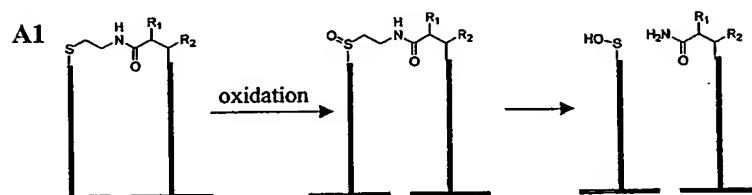
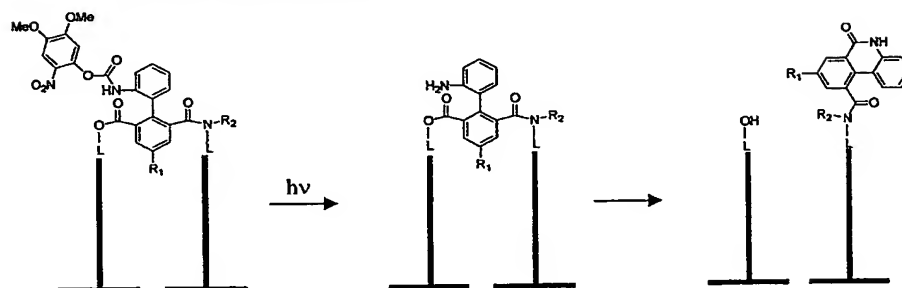
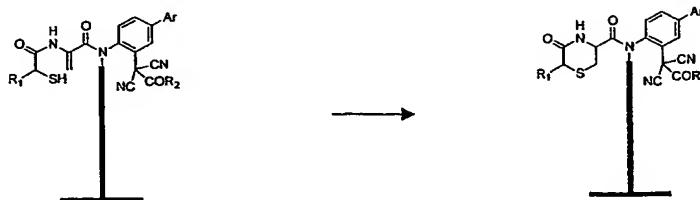
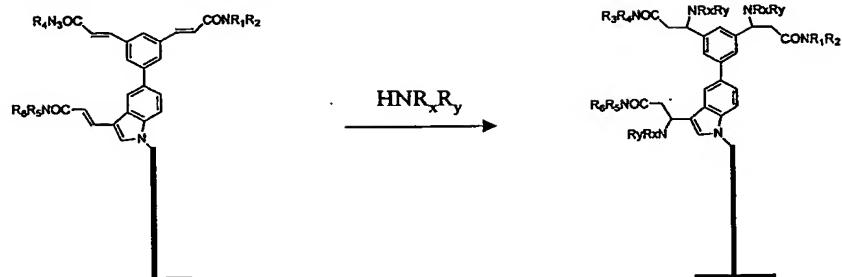
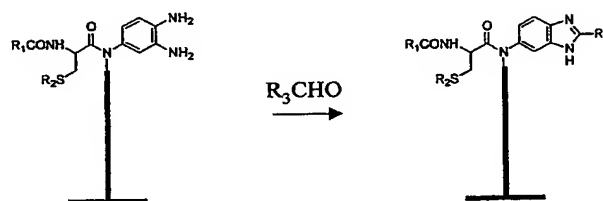
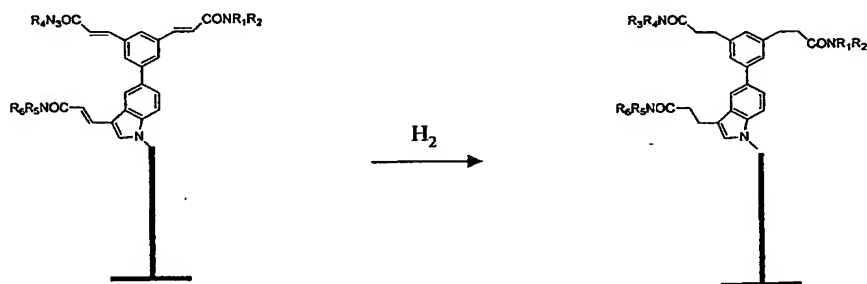
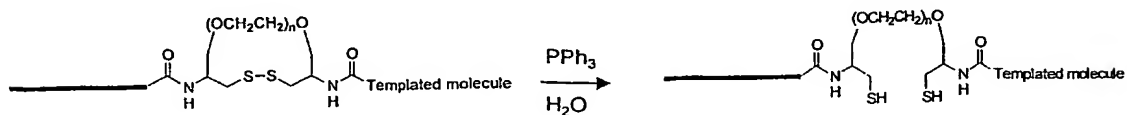
Fig. 26. Post-templating modification of templated molecule**A** Rearrangement and cleavage in one step, eg:**A2**

Photo labile protecting group

**B** Reaction of functional groups present in a templated molecule**B1** Intramolecular Michael addition:

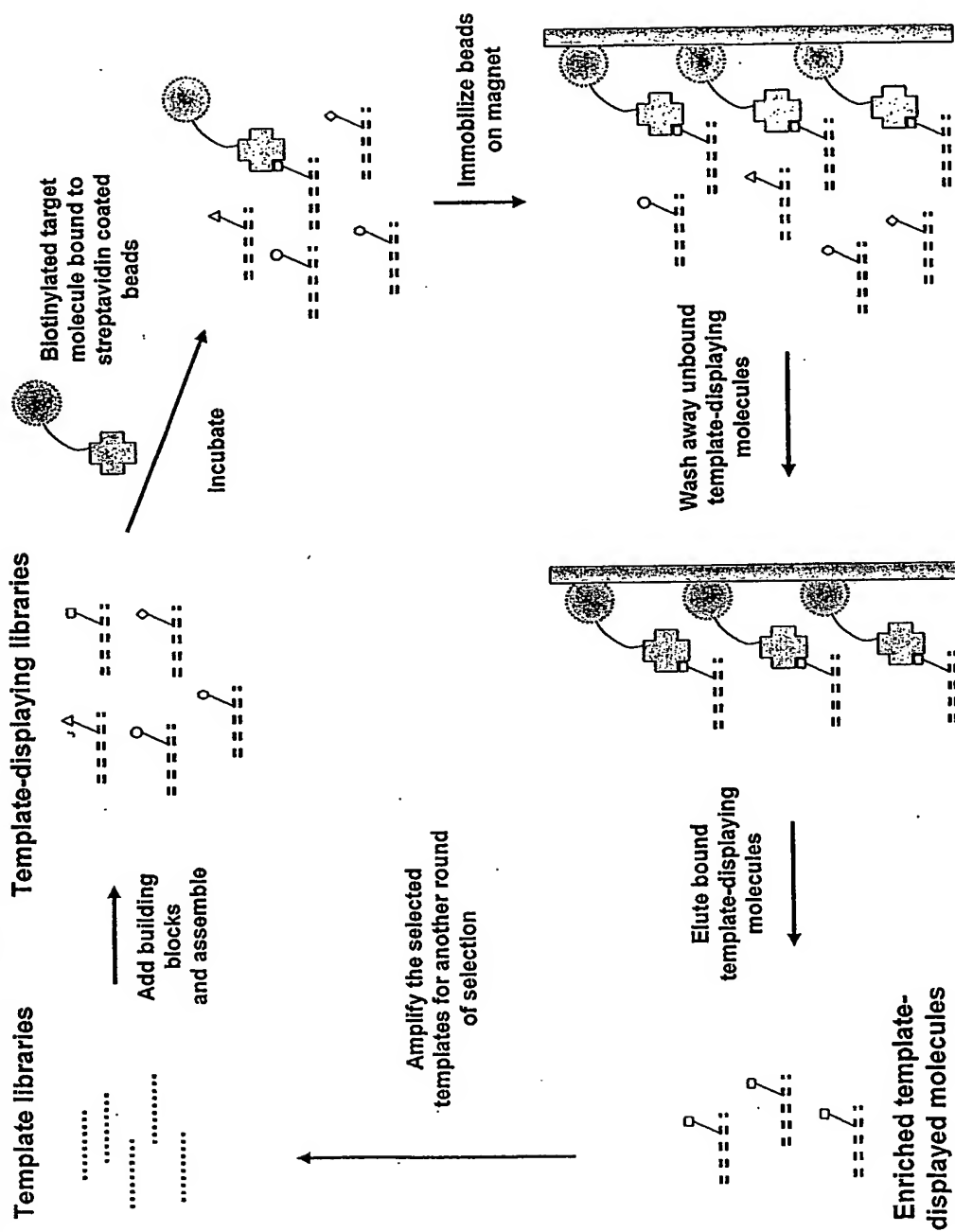
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B2 Intermolecular Michael addition:**B3 Reaction of phenylenediamines and aldehydes to form benzimidazoles:****B4 Reduction of multiple bonds:****C Post templating modification of linker to extend the spacing between the template and the templated molecule.**

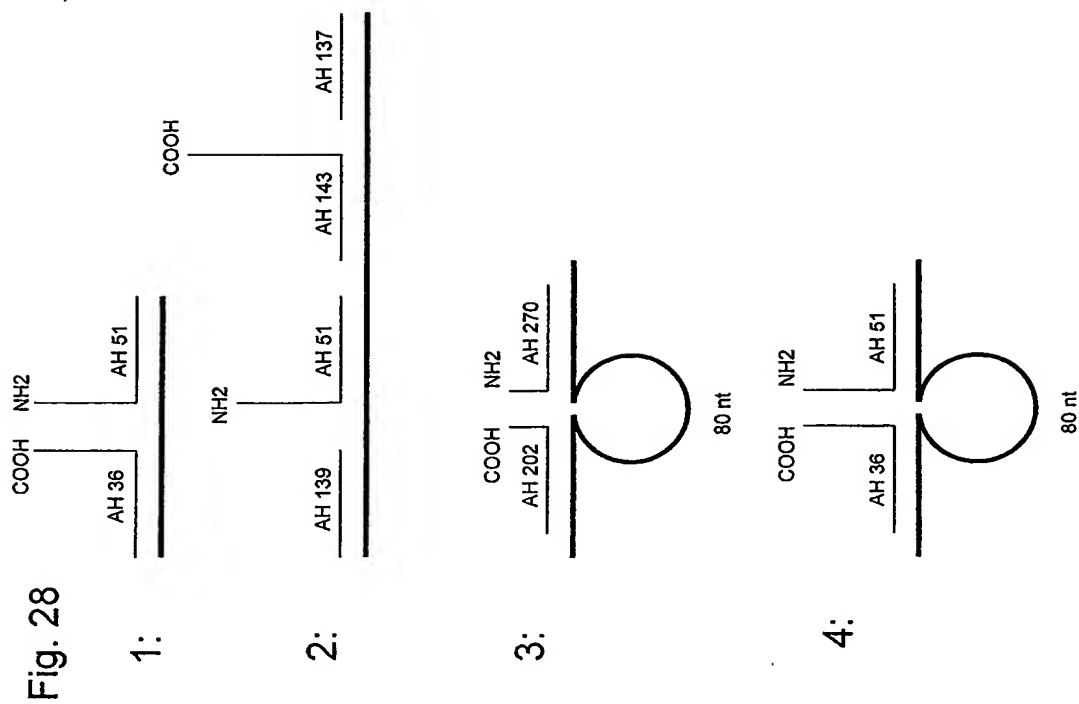
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Figure 27.
A typical panning protocol for selection of template-displaying molecules



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Reaction efficiency



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Reaction efficiency

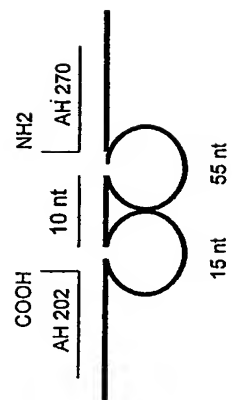
Fig. 28

(+)

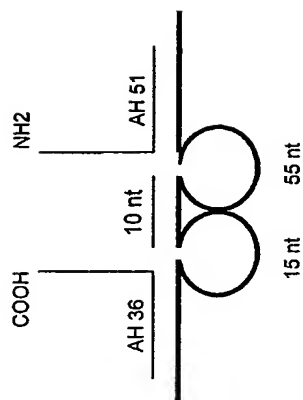
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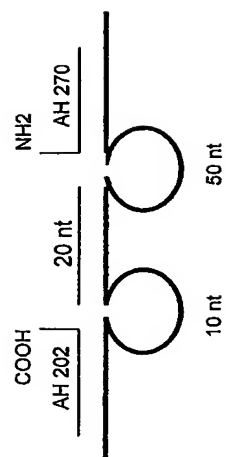
5:



6:



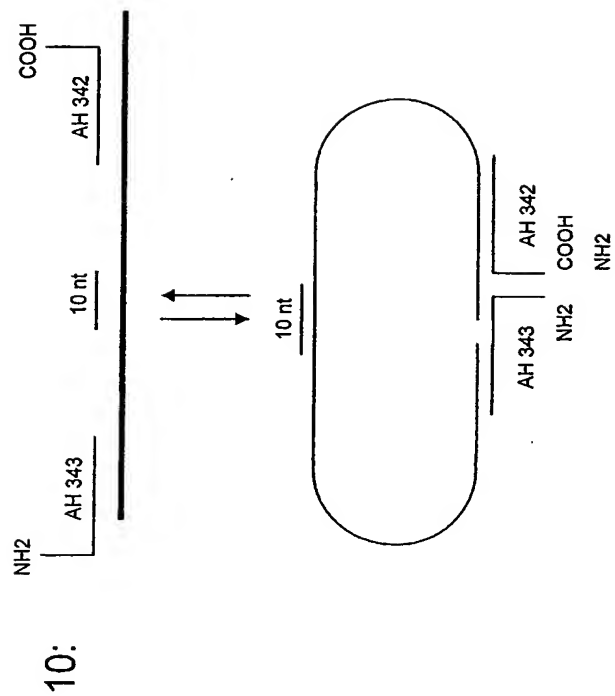
7:



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Reaction efficiency

Fig. 28

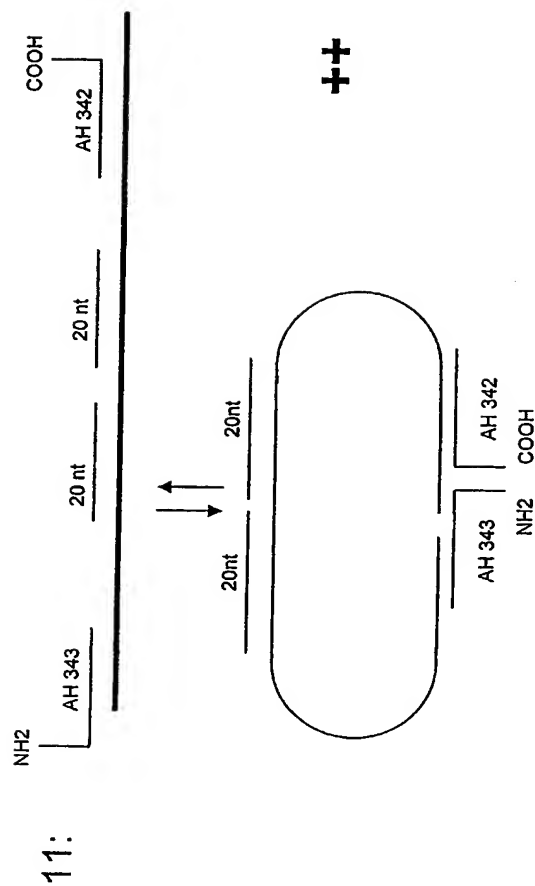


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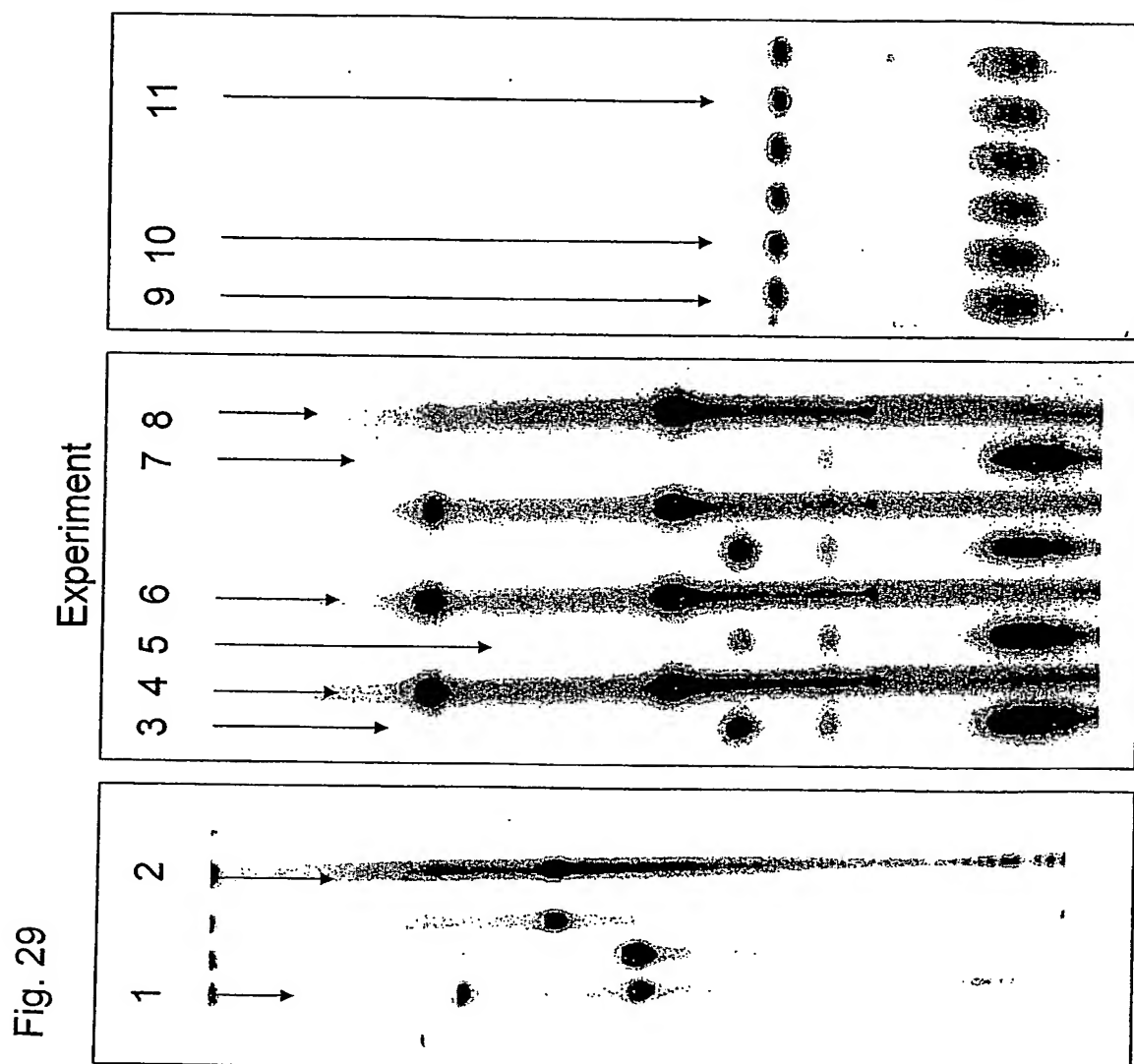
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Reaction efficiency

Fig. 28



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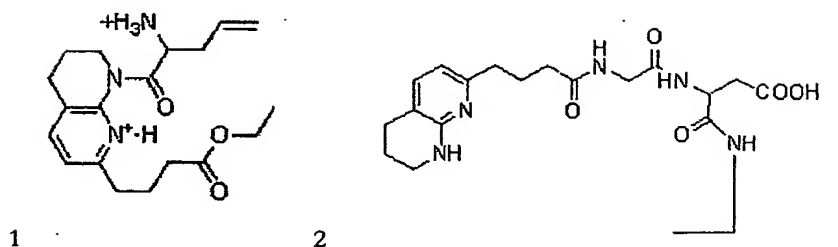


Figure 30. Structure 1 shows the Feuston 3 functional entity, which is needed together with Gly and Asp to create Feuston 5 structure 2, a ligand that binds to the $\alpha_v\beta_3$ integrin receptor (as described in press; Feuston BP et al. J Med Chem. 2002 Dec 19;45(26):5640-8)

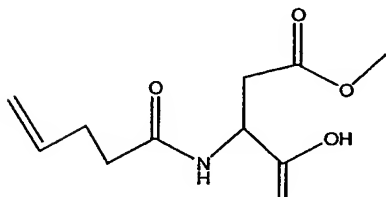


Figure 31. Structure of the pentenoyl protected aspartate functional entity used to load an amino modified scaffold oligo, to create the Feuston 5 ligand.

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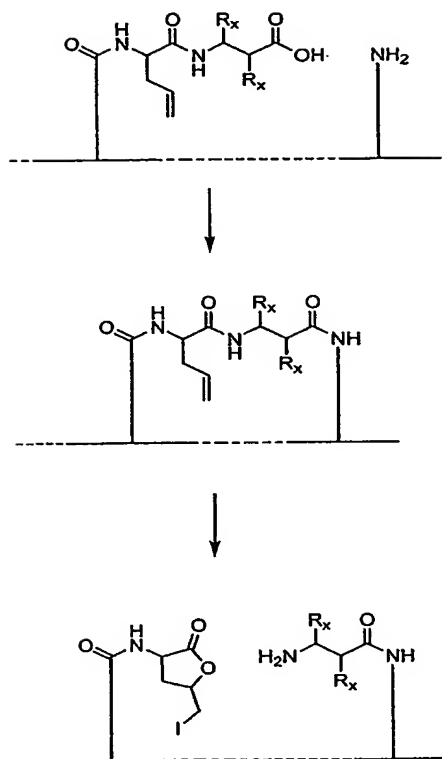


Figure 32. Allylglycine chemistry illustrated by structure showing cross-linked product as well as transferred product after cleavage by iodine.

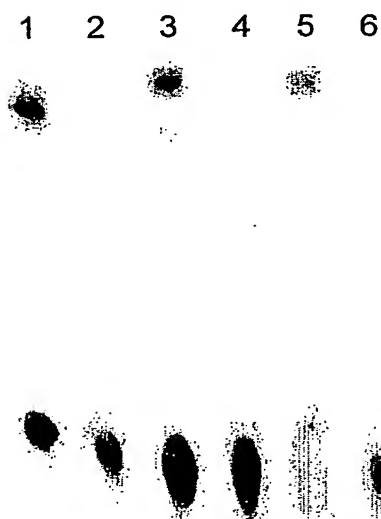


Figure 33. An autoradiography showing the three transfers of β -Ala to an amino modified scaffold oligo, this scaffold oligo being radioactively labeled. Lanes 1, 3 and 5 shows cross-linked product between scaffold amine and functional entity β -Ala AG carboxylic acid for transfers 1, 2 and 3. Lanes 2, 4 and 6 shows cleaved product, i.e. scaffold carrying the transferred functional entity.

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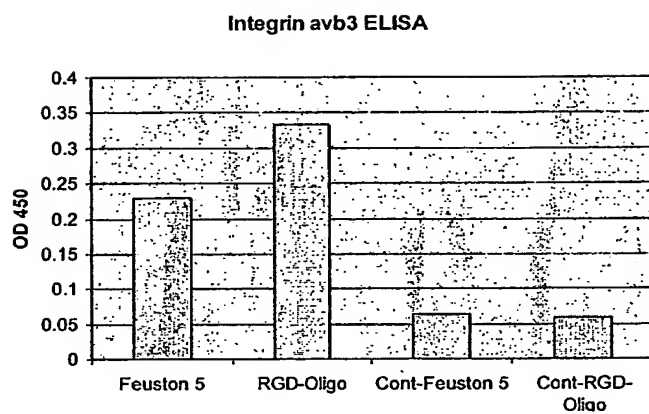


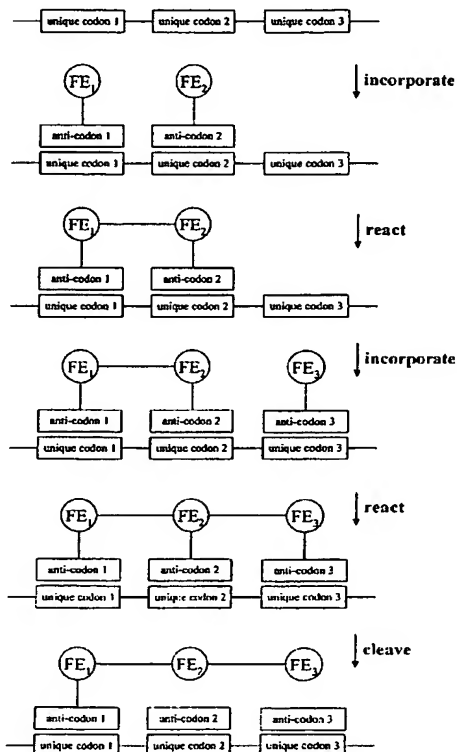
Figure 34 Result from the ELISA done on the feuston 5 ligand generated by sequential transfers to a scaffold oligo (first column). The controls are the RGD peptide, which is an Integrin ligand (second column;) loaded on a 20 mer oligo, and uncoated wells (no Integrin immobilized; third and fourth columns).



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PA 2002 01171 1 August 2002 (01.08.2002) DK
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- (71) Applicant (for all designated States except US): NUEVO-
LUTION A/S [DK/DK]; Rønnegade 8, 5th floor, DK-2100
Copenhagen Ø (DK).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): PEDERSEN, Henrik
[DK/DK]; Frodesvej 24, DK-2880 Bagsvaerd (DK).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM,
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European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report

[Continued on next page]

(54) Title: MULTI-STEP SYNTHESIS OF TEMPLATED MOLECULES



(57) Abstract: Disclosed is a method for the manufacture of a library of complexes. The complexes comprise templated molecules attached to the template which directed the synthesis thereof. The templated molecules are produced in a step-by-step fashion which provides for a high local concentration of reactive groups involved in the formation of connections between the individual components of the template molecule.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, MEDLINE, EMBASE, CHEM ABS Data, WPI Data, PAJ		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 02 074929 A (KANAN MATTEW W; GARTNER ZEV J ; LIU DAVID R (US); HARVARD COLLEGE () 26 September 2002 (2002-09-26) figures 1-57 ---	1-65
P,X	WO 02 103008 A (GOUILAEV ALEX HAAHR ; NOERREGAARD-MADSEN MAD S (DK); SLOEK FRANK ABI) 27 December 2002 (2002-12-27) figure 1 ---	1-65
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<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
* Special categories of cited documents : <div style="display: flex;"> <div style="flex: 1;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified).</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
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A	KEILER K C ET AL: "Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA." SCIENCE. UNITED STATES 16 FEB 1996, vol. 271, no. 5251, 16 February 1996 (1996-02-16), pages 990-993, XP002265223 ISSN: 0036-8075 figures 1-3 -----	1-65

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International Application No

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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: ENCODED MOLECULES BY TRANSLATION (EMT)

(57) Abstract: The present invention is directed to methods for the synthesis, selection, amplification and isolation of templated molecules having desirable properties. The invention makes it possible to synthesise a variety of different templated molecules other than α -peptides and modified α -peptides. In particular, the present invention enables synthesis of templated molecules such as β -, γ -, ω peptides, carbopeptoids, vinylogous peptides, oligoanthrilamides, oligoureas, azapeptides (azatides), oligocarbamates, PNA, oligopyrrolinones, vinylogous sulfonamidopeptide, peptoids, azapeptoids and hydrazino peptides. It is possible in accordance with the methods of the invention to synthesise templated molecules comprising heterocyclic components, such as coumarins and quinolones, pyrazolone, isoxazolone, pyrimidiones, phthalhydrazides, diketopiperazines, hydantoin, and benzodiazepines.

WO 2004/110964 A2

Encoded Molecules by Translation (EMT)

Technical Field of the Invention

- 5 The present invention relates to the field of polymers and branched molecules encoded by a template, i.e. templated molecules. Furthermore, the invention relates to methods of preparing encoded polymers and encoded branched molecules.

Background

10

One central dogma in biology describes the flow of information as a one-way process from DNA to RNA to polypeptide. DNA is transcribed by a RNA polymerase into mRNA; and the mRNA is subsequently then translated into protein by the ribosomes and tRNAs.

15

- The direct relation between DNA and protein, i.e., the fact that the sequence of triplet codons defines the sequence of α -amino acid residues in a polypeptide, has allowed the development of numerous molecular biological methods, wherein DNA may be manipulated (mutagenizes, recombines, deletes, inserts, etc), and then
20 used in in vivo systems (e.g., microbes) or in vitro systems (e.g., Zubay in vitro expression systems) to transfer the resulting changes from DNA level to the level of templated polypeptide, i.e., to mutate, recombine, delete, insert, etc. the polypeptide.

25

- Several systems have been invented that allows a flow of information from polypeptide to DNA the so-called retro-genetics. These systems include phage display, ribosome/polysome display, peptides-on-plasmid display, and other systems. These systems introduce a physical link between the template (e.g., DNA or RNA) and the templated polypeptide. As a result, it is possible, from a population
30 of templated polypeptides linked to their respective templates, to first enrich for a desired characteristic of the templated polypeptide (e.g., binding of the templated molecule to an affinity column), and subsequently amplify the enriched population of templated polypeptides through amplification of its template (DNA or RNA), followed by translation of the amplified templates. These methods have been used to identify

polypeptides with novel and/or improved features from libraries that may consist of more than 10^{14} polypeptides.

5 The critical feature of the prior art systems is the amplifiability of the templated molecule, through amplification of its template. Thus, after the selection step in which molecules with the desired property are enriched, the enriched population may be amplified and then taken through yet a selection step, etc. - the process of selection-and-amplification may be repeated many times. In this way the "noise" of the selection assay is averaged out over several selection-and-amplification rounds, 10 and even if the individual selection step yield only modest enrichment e.g. 10-fold, a theoretical enrichment of 10^{12} can be reached after 12 selection-and-amplification rounds.

In the field of chemistry, a different combinatorial approach has been developed. 15 This approach involved the parallel synthesis of millions of related compounds, in an array (where each position defined a specific compound), or on beads (where one bead carried many copies of the same compound). The population of compounds are then screened for desired characteristics. Importantly, this type of combinatorial library has no means for amplification, and therefore requires the use of very 20 stringent screening methods, as explained above.

Principles for tagging chemical libraries have also been developed. For example, systems that employed DNA oligos to tag molecule libraries have been developed as exemplified herein below. The tag is used as a means of identification, but cannot 25 be used to template the synthesis of the tagged molecule. Therefore, despite the tag, these systems still require a very efficient screening method.

Below prior art in the field of the invention is summarised:

30 WO 00/23458 describes combinatorial libraries, wherein each library component comprises a nucleic acid tag, which encodes the synthesis of a polymeric compound covalently attached thereto. The synthesis of the compound requires a "split and recombine" strategy. Hence, the synthesis can not be performed in one closed chamber, but requires that the library components are split according to the

sequence of their respective nucleic acid tags, before addition of a unit to the polymeric compound.

- 5 EP 0 604 552 B1 relates to a method for synthesizing diverse collections of oligomers. The invention involves the use of an identifier tag to identify the sequence of monomers in an oligomer. The identifier tags facilitate subsequent identification of reactions through which members of a library of different synthetic compounds have been synthesised in a component by component fashion.
- 10 EP 0 643 778 B1 relates to encoded combinatorial chemical libraries. Each of a collection of polypeptides is labelled by an appended "genetic" tag, itself constructed by chemical synthesis, to provide a "retro-genetic" way of specifying each polypeptide.
- 15 EP 0 773 227 A1 relates to a method for preparing a new pharmaceutical drug or diagnostic reagent, which includes the step of screening, against a ligand or receptor, a library of different synthetic compounds obtainable by synthesis in a component by component fashion.
- 20 US 4,863,857 relates to a method for determining the amino acid sequence of a polypeptide complementary to at least a portion of an original peptide or protein. In one aspect the method involves: (a) determining a first nucleotide sequence of a first nucleic acid coding for the biosynthesis of at least a portion of the original peptide or protein; (b) ascertaining a second nucleotide sequence of a second nucleic acid
25 which base-pairs with the first nucleotide sequence of the first nucleic acid, the first and second nucleic acids pairing in antiparallel directions; and (c) determining the amino acid sequence of the complementary polypeptide by the second nucleotide sequence when read in the same reading frame as the first nucleotide sequence.
- 30 US 5,162,218 relates to polypeptide compositions having a binding site specific for a particular target ligand and further having an active functionality proximate the binding site. The active functionality may be a reporter molecule, in which case the polypeptide compositions are useful in performing assays for the target ligand. Also disclosed are methods for preparing polypeptides having active functionalities
35 proximate their binding site, said method comprising the step of combining the

polypeptide specific for the target ligand with an affinity label having a reactive group attached thereto. The reactive group is then covalently attached to an amino acid side chain proximate the binding site and cleaved from the substrate. The substrate is subsequently eluted, leaving a moiety of the reactive group covalently attached to the polypeptide. The active functionality may then be attached to the moiety.

US 5,270,170 relates to a random peptide library constructed by transforming host cells with a collection of recombinant vectors that encode a fusion protein comprised of a DNA binding protein and a random peptide and also encode a binding site for the DNA binding protein. The fusion protein can be used for screening ligands. The screening method results in the formation of a complex comprising the fusion protein bound to a receptor through the random peptide ligand and to the recombinant DNA vector through the DNA binding protein.

US 5,574,141 relates to functionalized carrier materials for the simultaneous synthesis and direct labelling of oligonucleotides as primers for template-dependent enzymatic nucleic acid syntheses. The polymeric carriers are loaded with nucleic acid building blocks which in turn contain labelling groups or precursors thereof. The polymeric carrier loaded in this way serves as a solid or liquid phase for the assembly of oligonucleotides which can be used as primers for a template-dependent enzymatic nucleic acid synthesis such as in sequencing analysis or in the polymerase chain reaction (PCR).

US 5,573,905 relates to an encoded combinatorial chemical library comprising a plurality of bifunctional molecules having both a chemical polymer and an identifier oligonucleotide sequence that defines the structure of the chemical polymer. Also described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biologically active molecules in pre-selected binding interactions.

US 5,597,697 relates to a screening assay for inhibitors and activators of RNA and DNA-dependent nucleic acid polymerases. The invention provides methods for the identification and discovery of agents which are inhibitors and activators of RNA and DNA-dependent nucleic acid polymerases. The essential feature of the invention is the incorporation of a functional polymerase binding site sequence (PBS) into a

- nucleic acid molecule which is chosen for its ability to confer a discernible characteristic via its sequence specific activity such that the incorporation of the PBS renders the nucleic acid molecule a functional template for a predetermined RNA or DNA-template directed nucleic acid polymerase. In the presence of the polymerase, suitable primer molecules, and any necessary accessory molecules, catalytic extension of the strand of nucleic acids complementary to the template occurs, resulting in a partial or total elimination of (or increase in) the characteristic conferring activity of the reporter-template molecule described due to the antisense effects of the complementary strand or other polymerase-mediated effects.
- US 5,639,603 relates to a method for synthesizing and screening molecular diversity by means of a general stochastic method for synthesizing compounds. The method can be used to generate large collections of tagged compounds that can be screened to identify and isolate compounds with useful properties.
- US 5,698,685 relates to a morpholino-subunit combinatorial library and a method for generating a compound capable of interacting specifically with a selected macromolecular ligand. The method involves contacting the ligand with a combinatorial library of oligomers composed of morpholino subunits with a variety of nucleobase and non-nucleobase side chains. Oligomer molecules that bind specifically to the receptor are isolated and their sequence of base moieties is determined. Also disclosed is a combinatorial library of oligomers useful in the method and novel morpholino-subunit polymer compositions.
- US 5,708,153 relates to a method for synthesizing diverse collections of tagged compounds by means of a general stochastic method for synthesizing random oligomers on particles. A further aspect of the invention relates to the use of identification tags on the particles to facilitate identification of the sequence of the monomers in the oligomer.
- US 5,719,262 relates to a novel class of compounds, known as peptide nucleic acids, which bind complementary DNA and RNA strands more strongly than the corresponding DNA or RNA strands, and exhibit increased sequence specificity and solubility. The peptide nucleic acids comprise ligands selected from a group consisting of naturally-occurring nucleobases and non-naturally-occurring

nucleobases attached to a polyamide backbone, and contain alkylamine side chains.

5 US 5,721,099 relates to encoded combinatorial chemical libraries encoded with tags. Encoded combinatorial chemistry is provided, whereby sequential synthetic schemes are recorded using organic molecules, which define choice of reactant, and stage, as the same or different bit of information. Various products can be produced in the multi-stage synthesis, such as oligomers and synthetic non-repetitive organic molecules. Particularly, pluralities of identifiers may be used to
10 provide a binary or higher code, so as to define a plurality of choices with only a few detachable tags. The particles may be screened for a characteristic of interest, particularly binding affinity, where the products may be detached from the particle or retained on the particle. The reaction history of the particles which are positive for the characteristic can be determined by the release of the tags and analysis to
15 define the reaction history of the particle.

US 5,723,598 relates to an encoded combinatorial chemical library comprised of a plurality of bifunctional molecules having both a chemical polymer and an identifier oligonucleotide sequence that defines the structure of the chemical polymer. Also
20 described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biologically active molecules in preselected binding interactions.

US 5,770,358 relates to tagged synthetic oligomer libraries and a general stochastic
25 method for synthesizing random oligomers. The method can be used to synthesize compounds to screen for desired properties. The use of identification tags on the oligomers facilitates identification of oligomers with desired properties.

US 5,786,461 relates to peptide nucleic acids having amino acid side chains. A
30 novel class of compounds, known as peptide nucleic acids, bind complementary DNA and RNA strands more strongly than the corresponding DNA or RNA strands, and exhibit increased sequence specificity and solubility. The peptide nucleic acids comprise ligands selected from a group consisting of naturally-occurring nucleobases and non-naturally-occurring nucleobases attached to a polyamide
35 backbone, and contain alkylamine side chains.

US 5,789,162 relates to a method for synthesizing diverse collections of oligomers. A general stochastic method for synthesizing random oligomers on particles is disclosed. A further aspect of the invention relates to the use of identification tags on the particles to facilitate identification of the sequence of the monomers in the oligomer.

US 5,840,485 relates to topologically segregated, encoded solid phase libraries. Libraries of synthetic test compounds are attached to separate phase synthesis supports that also contain coding molecules that encode the structure of the synthetic test compound. The molecules may be polymers or multiple nonpolymeric molecules. The synthetic test compound can have backbone structures with linkages such as amide, urea, carbamate (i.e., urethane), ester, amino, sulfide, disulfide, or carbon-carbon, such as alkane and alkene, or any combination thereof. The synthetic test compound can also be molecular scaffolds, or other structures capable of acting as a scaffold. The invention also relates to methods of synthesizing such libraries and the use of such libraries to identify and characterize molecules of interest from among the library of synthetic test compounds.

US 5,843,701 relates to systematic polypeptide evolution by reverse translation and a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

US 5,846,839 relates to a method for hard-tagging an encoded synthetic library. Disclosed are chemical encryption methods for determining the structure of compounds formed in situ on solid supports by the use of specific amine tags which, after compound synthesis, can be deencrypted to provide the structure of the compound found on the support.

US 5,922,545 relates to methods and compositions for identifying peptides and single-chain antibodies that bind to predetermined receptors or epitopes. Such

peptides and antibodies are identified by methods for affinity screening of polysomes displaying nascent peptides.

5 US 5,958,703 relates to methods for screening libraries of complexes for compounds having a desired property such as the capacity to bind to a cellular receptor. The complexes in such libraries comprise a compound under test, a tag recording at least one step in synthesis of the compound, and a tether susceptible to modification by a reporter molecule. Modification of the tether is used to signify that a complex contains a compound having a desired property. The tag can be decoded
10 to reveal at least one step in the synthesis of such a compound

US 5,986,053 relates peptide nucleic acid complexes of two peptide nucleic acid strands and one nucleic acid strand. Peptide nucleic acids and analogues of peptide nucleic acids are used to form duplex, triplex, and other structures with nucleic acids
15 and to modify nucleic acids. The peptide nucleic acids and analogues thereof also are used to modulate protein activity through, for example, transcription arrest, transcription initiation, and site specific cleavage of nucleic acids.

US 5,998,140 relates to methods and compositions for forming complexes
20 intracellularly between dsDNA and oligomers of heterocycles, aliphatic amino acids, particularly omega-amino acids, and a polar end group. By appropriate choice of target sequences and composition of the oligomers, complexes are obtained with low dissociation constants.

25 US 6,060,596 relates to an encoded combinatorial chemical library comprised of a plurality of bifunctional molecules having both a chemical polymer and an identifier oligonucleotide sequence that defines the structure of the chemical polymer. Also described are the bifunctional molecules of the library, and methods of using the library to identify chemical structures within the library that bind to biologically active
30 molecules in preselected binding interactions.

US 6,080,826 relates to Template-directed ring-closing metathesis and ring-opening metathesis polymerization of functionalized dienes. Functionalized cyclic olefins and methods for making the same are disclosed. Methods include template-directed
35 ring-closing metathesis ("RCM") of functionalized acyclic dienes and template-

directed depolymerization of functionalized polymers possessing regularly spaced sites of unsaturation. Although the template species may be any anion, cation, or dipolar compound, cationic species, especially alkali metals, are preferred.

5 Functionalized polymers with regularly spaced sites of unsaturation and methods for making the same are also disclosed. One method for synthesizing these polymers is by ring-opening metathesis polymerization ("ROMP") of functionalized cyclic olefins.

10 US 6,127,154 relates to compounds which possess a complementary structure to a desired molecule, such as a biomolecule, in particular polymeric or oligomeric compounds, which are useful as in vivo or in vitro diagnostic and therapeutic agents are provided. Also, various methods for producing such compounds are provided.

15 US 6,140,493 relates to a method for synthesizing diverse collections of oligomers. A general stochastic method for synthesizing random oligomers is disclosed and can be used to synthesize compounds to screen for desired properties. Identification tags on the oligomers facilitates identification of oligomers with desired properties.

20 US 6,140,496 relates to building blocks for preparing oligonucleotides carrying non-standard nucleobases that can pair with complementary non-standard nucleobases so as to fit the Watson-Crick geometry. The resulting base pair joins a monocyclic six membered ring pairing with a fused bicyclic heterocyclic ring system composed of a five member ring fused with a six member ring, with the orientation of the heterocycles with respect to each other and with respect to the backbone chain analogous to that found in DNA and RNA, but with a pattern of hydrogen bonds
25 holding the base pair together different from that found in the AT and GC base pairs (a "non-standard base pair").

30 US 6,143,497 relates to a method for synthesizing diverse collections of random oligomers on particles by means of a general stochastic method. Also disclosed are identification tags located on the particles and used to facilitate identification of the sequence of the monomers in the oligomer.

35 US 6,165,717 relates to a general stochastic method for synthesizing random oligomers on particles. Also disclosed are identification tags located on the particles to facilitate identification of the sequence of the monomers in the oligomer.

US 6,175,001 relates to functionalized pyrimidine nucleosides and nucleotides and DNA's incorporating same. The modified pyrimidine nucleotides are derivatized at C5 to contain a functional group that mimics the property of standard amino acid residues. DNA molecules containing the modified nucleotides are also provided.

US 6,194,550 B1 relates to systematic polypeptide evolution by reverse translation, in particular a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

US 6,207,446 B1 relates to methods and reagents for the selection of protein molecules that make use of RNA-protein fusions.

US 6,214,553 B1 relates to methods and reagents for the selection of protein molecules that make use of RNA-protein fusions.

WO 91/05058 relates to a method for the cell-free synthesis and isolation of novel genes and polypeptides. An expression unit is constructed onto which semi-random nucleotide sequences are attached. The semi-random nucleotide sequences are first transcribed to produce RNA, and then translated under conditions such that polysomes are produced. Polysomes which bind to a substance of interest are then isolated and disrupted; and the released mRNA is recovered. The mRNA is used to construct cDNA which is expressed to produce novel polypeptides.

WO 92/02536 relates to a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

WO 93/03172 relates to a method for preparing polypeptide ligands of target molecules wherein candidate mixtures comprised of ribosome complexes or

mRNA:polypeptide copolymers are partitioned relative to their affinity to the target and amplified to create a new candidate mixture enriched in ribosome complexes or mRNA:polypeptide copolymers with an affinity to the target.

- 5 WO 93/06121 relates to a general stochastic method for synthesizing random oligomers on particles. Also disclosed are identification tags located on the particles to facilitate identification of the sequence of the monomers in the oligomer.

- 10 WO 00/47775 relates to a method for generating RNA-protein fusions involving a high-salt post-translational step.

- Additional references of relevance for present invention includes Bain et al. Nature, vol. 356, 1992, 537-539; Barbas et al. Chem. Int. Ed. vol. 37, 1998, 2872-2875; Benner Reviews; Blanco et al. Analytical Biochemistry vol. 163, 1987, 537-545; 15 Brenner et al. Proc. Natl. Acad. Sci. Vol. 89, 1992, 5381-5383; Bresler et al. Biochimica et Biophysica Acta vol. 155, 1968, 465-475; Dewey et al. J. Am. Chem. Soc. Vol. 117, 1995, 8474-8475; Dietz et al. Photochemistry and photobiology vol. 49, 1989, 121-129; Gryaznov et al. J. Am. Chem. Soc. vol. 115, 1993, 3808-3809; Gryaznov et al. Nucleic Acids Research vol. 21, 1993, 1403-1408; Elmar Gocke 20 Mutation Research vol. 248, 1991, 135-143; Haeuptle et al. Nucleic Acids Research, 14, 1986, 1427-1448; Hamburger et al. Biochimica et Biophysica Acta, 213, 1970, 115-123; Hamza A. El-Dorry Biochimica et Biophysica Acta vol. 867, 1986, 252-255; Herrera-Estrella et al. The EMBO Journal, 7, 1988, 4055-4062; Heywood et al. Biochemistry vol. 57, 1967, 1002-1009; Heywood et al. J. Biol. Chem. Vol. 7, 1968, 25 3289-3296; Hooper et al. Eur. J. Clin. Microbiol. Infect. Dis. Vol. 10, 1991, 223-231; Houdebine et al. Eur. J. Biochem., 63, 1976, 9-14; Johnson et al. Biochemistry vol. 25, 1986, 5518-5525; Kinoshita et al. Nucleic Acids Symposium Series vol. 34, 1995, 201-202; Leon et al. Biochemistry vol. 26, 1987, 7113-7121; Maclean et al. Proc. Natl. Acad. Sci. USA vol. 94, 1997, 2805-2810; Mattheakis et al. Proc. Natl. 30 Acad. Sci. USA vol. 91, 1994, 9022-9026; Menninger et al. Antimicrobial Agents and Chemotherapy, 21, 1982, 811-818; Menninger. Biochimica et Biophysica Acta, 240, 1971, 237-243; Mirzabekov Methods in Enzymology vol. 170, 1989, 386-408; Nikolaev et al. Nucleic Acids Research vol. 16, 1988, 519-535; Noren et al. Science vol. 24, 1989, 182-188; Pashev et al. TIBS vol. 16, 1991, 323-326; Pargellis et al. 35 The Journal of Biological Chemistry, 263, 1988, 7678-7685; Pansegrau et al. The

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Poly-peptide synthesis by ribosomes involves peptidyl transferase activity coordinating the interaction of a carboxy group of an α -amino acid in the ribosomal P-site and the amino group of an α -amino acid in the A-site forming an amide bond. Successive additions of α -amino acids results in formation of an α -peptide (protein). Each amino acid is presented in the ribosome by an amino acid-specific tRNA that read (decode) the template mRNA. The sequence of an mRNA is "decoded" in increments of three nucleotides (a codon) specifying a single amino-acid. Consequently, a nucleotide diversity of 4 (G, A, U, C) in RNA and a triple nucleotide (codon) read-out will produce 64 different codon sequences each corresponding to an α -amino acid or protein factors necessary for translation termination. In almost all natural systems only 20 amino acids specified by 61 codons are incorporated into proteins. Though nature only employs 20 standard amino acids many non-standard amino acids (NS-AA) and pseudo-amino acids have been incorporated into proteins both in vitro and in vivo (Liu et al 1999). A non-exhaustive list of non-standard amino acids and pseudo amino acids incorporated into proteins during the process of translation is shown in figure 3.

The chemical diversity of amino acid residues provide a valuable tool for the synthesis of a random or semi-random library of peptide molecules using in vitro or in situ translation (and transcription) in extracts of prokaryotic or eukaryotic origin. From such libraries it is possible to select molecules of desired functionality based on the interaction with a target of interest (for example an affinity column). However, when operating large libraries of for example 10^{14} different molecules it is practically impossible to select and identify single molecules of desired properties in a single step. In order to isolate specific peptide sequences with relevant characteristics, it is necessary to include a "retro-genetic element" preferably a DNA or RNA sequence, that templates the synthesis of the peptide sequence. This retro-genetic element enable amplification of a nucleotide sequence that encodes a specific peptide sequence having relevant properties. Furthermore, the retro-genetic element allows multiple rounds of selection and amplification of peptide sequences necessary for the isolation of unique peptides from large libraries. Consequently, several procedures have been developed that allows the formation of nucleic acid-peptide complexes formed by either covalent or non-covalent coupling between a peptide and the RNA or DNA that encode said peptide.

Peptide Display (SPERT – Systematic Polypeptide Evolution by Reverse Translation) a protocol acknowledged by those skilled in the art, describes peptide synthesis by ribosomes and the formation of ribosome-mRNA-peptide complexes by incomplete peptide synthesis (i.e. ribosome stalling). Ribosome-mRNA-peptide complexes can be partitioned based on peptide functionality allowing enrichment of complexes with desired properties. Subsequent amplification using reverse transcription and the polymerase chain reaction (PCR) of co-selected RNA sequences permits multiple selection and amplification rounds until a pool of peptide products having desired characteristics are obtained (patent no.: US.6,194,550 B1).

PROFusion, a protocol acknowledged by those skilled in the art, describes the covalent attachment of a peptide to the 3'-end of the mRNA which encodes said peptide (Roberts and Szostak, 1997). This protocol enables the synthesis of a library of RNA-peptide complexes that can be used for selection and isolation of peptide molecules with desired properties. Subsequently, the protein sequence information is recovered by amplification of the appended mRNA molecule using reverse transcription and PCR or equivalent techniques. The RNA-protein fusions

permit repeated rounds of selection and amplification allowing enrichment of peptides with relevant characteristics (patent no.: US 6,214,553 B1).

RIDS (Ribosome Inactivation Display System), is a protocol describing the connection between genotype and phenotype by formation of a stable complex between a ribosome, an mRNA and its translated protein. The coupling is based on the translation of the ricin A chain which enables cis-inactivation of the ribosome. Thus, predetermined sequences for library synthesis by translation are fused to the ricin A gene. Following synthesis of the peptide-ricin A fusion products the ribosome is inactivated forming stable ribosome-mRNA-peptide ternary complexes enabling selection of peptides having desired properties. Following selection, the appended genetic sequence of is amplified by reverse transcription and PCR. The RNA-fusion product permits repeated rounds of selection and amplification allowing enrichment of peptides with relevant properties (Zhou et al J. Am. Chem. Soc. 2002. in press.

Covalent Display Technology, a protocol describing the coupling of a DNA template and the peptide encoded by said DNA template. A DNA template comprising predetermined sequences fused to the *p2A* gene sequence of *E.coli* bacteriophage T2. Following transcription and translation each fusion peptide product produces a covalent attachment between said peptides and the DNA template that specifies the p2A fusion product. Thus, library of peptide sequences fused to their corresponding DNA templates can be used for selection of relevant peptides and subsequent amplification of the appended DNA template by polymerase chain reaction. Multiple rounds of selection and amplification allows for the isolation of peptide sequences with desired properties.

Summary of the Invention

A central process in biology is the formation of polypeptides involving ribosome mediated translation of an RNA template (mRNA). This process converts the genetic information encoded by mRNAs into specific sequences of alpha-amino-acids forming the polypeptides (proteins) that perform nearly all biological processes within a living cell.

Hence, biological systems allow template-directed synthesis of alpha-peptides by the process of ribosome mediated translation of a messenger-RNA (mRNA) template. The present invention describes a system that allows template-directed synthesis of other types of polymers and branched molecules in addition to alpha-peptides. Polymers or branched molecules synthesised by template-directed synthesis (see definition herein below) are designated templated molecules throughout the description.

Templated molecules comprise a plurality of functional groups that are linked together forming a polymer or a branched molecule. Each functional group is usually initially linked to an amino acid or amino acid-like entity (non-standard amino acid or pseudo amino acid) and thus constitutes a non-standard sidegroup of said amino acid or amino acid-like entity. Upon translation of a predetermined mRNA template the incorporation of amino-acids carrying non-standard sidegroups produce peptides carrying appended functional entities. The appended functional entities can subsequently be linked to adjacent functional entities resulting in the formation of a templated molecule linked to its template.

The present invention describes methods to synthesise, select, amplify and isolate templated molecules of desired properties. In particular, the invention overcomes some of the inherent limitations of the technologies described in the prior art, thereby allowing for the synthesis of a variety of different templated molecules that are not limited to α -peptides or modified α -peptides. The present invention enables the synthesis of templated molecules such as β -, γ -, ω peptides, carbopeptoids, vinylogous peptides, oligoanthrilamides, oligoureas, azapeptides (azatides), oligocarbamates, PNA, oligopyrrolinones, vinylogous sulfonamidopeptide, peptoids, azapeptoids or hydrazino peptides. Furthermore it is possible to synthesise templated molecules comprising heterocyclic components for example coumarins and quinolones, pyrazolone, isoxazolone, pyrimidiones, phthalhydrazides, diketopiperazines, hydantoins and benzodiazepines.

Thus, the invention relates to a method for templating molecules. The invention also relates to a method for covalent or non-covalent coupling between a template and templated molecule that in preferred embodiments enable amplification of the templated molecule by amplification of the template encoding it.

5 The system combines the advantages of the natural system (information flow from template to templated molecule), as well as the recently invented ribosome-mediated systems (e.g., PROFusion), namely the physical link between template and the templated molecule.

10 Accordingly, it is a first objective of the present invention to provide methods for synthesising a templated molecule comprising a plurality of functional groups, said method comprising the steps of

- 15 i) providing at least one template comprising a sequence of n coding elements selected from the group consisting of first coding elements and second coding elements,
- wherein each coding element comprises at least one recognition group capable of recognising a predetermined complementing element, and
- wherein n is an integer of at least 3,
- 20 with the proviso that the template comprises at least 3 first coding elements,
- 25 ii) providing a plurality of building blocks selected from the group consisting of first building blocks and second building blocks, with the proviso that at least 3 first building blocks are provided,
- wherein each first building block comprises
- 30 a) at least one complementing entity comprising a first complementing element comprising at least one recognition group capable of recognising a predetermined first coding element,
- b) at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and

35

- c) at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity, and

5 wherein each second building block comprises

- a) at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,

10

- b) and at least one spacer comprising at least one spacer reactive group,

15

complementing coding elements by contacting each coding element with a building block complementing element capable of recognising said coding element, wherein at least 2 coding elements are interacting with complementing elements simultaneously,

20

with the proviso that a total of at least 3 first coding elements are complemented; and

25

forming a spacer backbone by linking neighbouring spacers in a ribosome catalysed reaction, by means of reacting spacer reactive groups, and

30

obtaining a templated molecule comprising at least 3 covalently linked, functional groups by linking, by means of reacting functional entity reactive groups, a functional group of one functional entity to a functional group of another adjacently positioned functional entity and linking said other functional entity to yet another adjacently positioned functional entity.

35

In one embodiment the present invention relates to the formation of branched molecules comprising a scaffold capable of forming at least 3 covalent bonds to functional groups by means of reactions between reactive groups on the scaffold

and reactive groups of at least 3 functional entities. Formation of branched molecules enables the formation of;

- 1) Mono, di, tri and oligo functional open-chain hydrocarbons.
- 2) Mono, di, tri and oligofunctional non-aromatic carbocycles.
5 Monocyclic, bicyclic, tricyclic and polycyclic hydrocarbons. Bridged polycyclic hydrocarbons.
- 3) Mono, di, tri, and oligofunctional non-aromatic heterocycles. Mono, bi, tri and polycyclic heterocycles. Bridged polycyclic heterocycles.
- 4) Mono, di, tri and oligo functional aromatic carbocycles. Mono, bi,
10 tri and polycyclic aromatic carbocycles.
- 5) Mono, di, tri and oligofunctional aromatic heterocycles. Mono, bi, tri and polycyclic heterocycles
- 6) Chelates.
- 7) Fullerenes
- 15 8) Any combination of the above.

20 It is a second objective of the present invention to provide templated molecules covalently linked to the template encoding said templated molecule, wherein said templated molecule comprises at least 3 covalently linked functional groups, each encoded by a coding element of said template, with the proviso, that the templated molecule is not an α -polypeptide.

25 It is a third objective of the present invention to provide a plurality of templated molecules, wherein the plurality comprises at least 1000 different templated molecules and wherein said templated molecule comprises at least 3 covalently linked, functional groups, each encoded by a coding element of a template, with the proviso, that the templated molecule is not an α -polypeptide.

30 Definitions

Activation

Activation of a templated molecule involves cleaving one or more of the cleavable linkers that connect functional entities to the spacer backbone.

Adjacently positioned

5 The adjacently positioned functional entities of any given functional entity are the functional entities, which are either closest to said given functional entity in 3 dimensional space or which are most likely to react chemically with the given functional entity.

10 Preferably, adjacently positioned functional entities of any given functional entity are the functional entities, which are most likely to react with said functional entity. Often this will be the functional entity that are closest to said functional entity in 3 dimensional space. Preferably the distance between 2 adjacently positioned functional entities is on average less than 50 Å, even more preferably less than 20 Å and most preferably less than 10 Å.

15 In one preferred embodiment adjacently positioned refers to two functional entities that are neighbouring on the spacer backbone.

Amino acid residue

20 The term "amino acid residue" is meant to encompass amino acids, either standard amino acids, non-standard amino acids or pseudo-amino acids, which have been reacted with at least one other species, such as 2, for example 3, such as more than 3 other species.

25 In particular amino acid residues may comprise an acyl bond in place of a free carboxyl group and/or an amine-bond and/or amide bond in place of a free amine group. Furthermore, reacted amino acids residues may comprise an ester or thioester bond in place of an amide bond

30 Amplification according to the present invention is the process wherein a plurality of exact copies of a starting molecule is synthesised, without employing knowledge of the exact composition of the starting molecule. Hence a template may be amplified even though the exact composition of said template is unknown.

In one preferred embodiment of the present invention amplification of a template comprises the process wherein a template is copied by a nucleic acid polymerase or polymerase homologue, for example a DNA polymerase or an RNA polymerase. For example, templates may be amplified using reverse transcription, the polymerase chain reaction (PCR), ligase chain reaction (LCR), in vivo amplification of cloned DNA, and similar procedures capable of complementing a nucleic acid sequence.

Anticodon

An anticodon is a sequence of 3 ribonucleotides that can pair with the bases of a corresponding codon on a messenger RNA.

In certain aspects of the invention it may be favourable to design anticodons that comprise more than 3 nucleotides, such as 4 ribonucleotides, such as 5 ribonucleotides, such as 6 ribonucleotides, such as more than 6 ribonucleotides, for example 8 ribonucleotides or for example 10 ribonucleotides.

Building block

Building blocks according to the present invention may be selected from the group consisting of first building blocks and second building blocks.

A first building block according to the present invention comprises:

at least one complementing entity comprising a first complementing element comprising at least one recognition group capable of recognising a predetermined first coding element,

at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and

at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity.

Preferably, the functional entity is separated from the spacer by a cleavable linker.

A specific example of a first building block is depicted in figure 4A.

5 A second building block according to the present invention comprises:

at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,

10

and at least one spacer comprising at least one spacer reactive group.

A specific example of a second building block is depicted in figure 4B.

15 Charging

Charging is the step in the synthesis of a first or a second building block, in which a spacer is coupled to a complementing entity. In a preferred aspect this refers to addition of FE-AA, standard amino acid, non-standard amino acid, pseudo-amino acids or precursors thereof to a tRNA or pre-tRNA or tRNA like structure by a chemical or enzymatic reaction.

20

Hence, the term "charged tRNA" refers to a tRNA covalently attached to a spacer, such as a FE-AA, standard amino acid, non-standard amino acid, pseudo-amino acids or precursors thereof.

25

Cleavable linker

A cleavable linker according to the present invention is a residue or chemical bond capable of being cleaved under specific predetermined conditions. Preferably, cleavable linkers are separating a spacer and a functional entity of a first building block.

30

Non-limiting examples of cleavable linkers that may be employed with the present invention are given in figure 10.

5 Coding elements

Coding elements according to the present invention comprises at least one recognition group capable of recognising a predetermined complementing element. Preferably, one particular coding element is capable of specifically interacting with
10 the predetermined complementing element, and accordingly the coding element preferably is not capable of interacting with other complementing elements, or interacts with less efficiency with these other complementing elements.

Coding elements may be selected from the group consisting of first coding elements
15 and second coding elements.

First coding element comprises at least one recognition group capable of recognising a predetermined first complementing element.

20 Second coding element comprises at least one recognition group capable of recognising a predetermined second complementing element.

In one preferred embodiment of the present invention the coding elements are selected from the group consisting of nucleotides, nucleotide derivatives, nucleotide
25 analogues, and any combination thereof, such as oligonucleotides or oligonucleotide analogues. Preferably, nucleotides are ribonucleic acids comprising a base selected from adenine (A), uracil (U), guanine (G), and cytosine (C) and derivatives and analogues thereof.

30 Each coding element may consists of any desirable number of nucleotides, nucleotide derivatives and/or nucleotide analogues, for example 1, such as 2, for example 3, such as 4, for example 5, such as 5 to 10, for example 10 to 15, such as 15 to 20, for example more than 20 nucleotides, nucleotide derivatives and/or nucleotide analogues. Preferably, each coding element consists of 3 of nucleotides,

nucleotide derivatives and/or nucleotide analogues. More preferably, the coding element is a codon.

- 5 Coding elements according to the present invention must be capable of serving as a template for a ribosome mediated incorporation of subunits into a polypeptide, a polypeptide derivative or a polypeptide analogue.

Codon

- 10 A codon is a sequence of 3 ribonucleotides that encodes a particular amino acid in a messenger RNA molecule.

Complementing entity

- 15 A complementing entity according to the present invention is an entity that comprises one complementing element. Preferably a complementing entity according to the present invention comprises only one complementing element.

- 20 In one preferred embodiment of the present invention the complementing element comprises and even more preferably consists of nucleotides and/or nucleotide analogues. For example nucleotides and nucleotide analogues selected from the group consisting of DNA, RNA, LNA, PNA and mixtures thereof.

- 25 In one preferred embodiment of the present invention the complementing entity is a tRNA or tRNA-like structure. By tRNA like structure is meant any structure, which can perform the function of tRNA, that is transfer of a standard amino acid, non-standard amino acid or pseudo amino acid to a template for ribosome mediated synthesis of a polypeptide, a polypeptide derivative or a polypeptide analogue.

- 30 In one embodiment of the present invention the complementing entity is a tRNA. In another embodiment of present invention the complementing entity is a pseudoknot.

Complementing element

- Complementing elements according to the present invention comprise at least one recognition group capable of recognising a predetermined coding element.
- 5 Preferably, one particular complementing element is capable of specifically interacting with the predetermined coding element, and accordingly the complementing element preferably is not capable of interacting with other coding elements, or interacts with less efficiency with these other coding elements.
- 10 A complementing element according to the present invention may be selected from the group consisting of first complementing elements and second complementing elements
- 15 First complementing elements according to the present invention comprise at least one recognition group capable of recognising a predetermined first coding element. When comprised within a building block, a first complementing element is comprised within a first building block.
- 20 Second complementing elements according to the present invention comprise at least one recognition group capable of recognising a predetermined second coding element. When comprised within a building block, a second complementing element is comprised within a second building block.
- 25 In one preferred embodiment of the present invention the complementing elements are selected from the group consisting of nucleotides, nucleotide derivatives, nucleotide analogues, and any combination thereof, such as oligonucleotides or oligonucleotide analogues.
- 30 Each complementing element may consist of any desirable number of nucleotides, nucleotide derivatives and/or nucleotide analogues. For example each complementing element may consist of 1, such as 2, for example 3, such as 4, for example 5, such as 5 to 10, for example 10 to 15, such as 15 to 20, for example more than 20 nucleotides, nucleotide derivatives and/or nucleotide analogues.

Preferably, the nucleotides may be selected from the group consisting of ribonucleic acids comprising a base selected from adenine (A), uracil (U), guanine (G), and cytosine (C) and derivatives and analogues thereof.

- 5 In one embodiment the complementing element may be an anticodon.

Decoding

- 10 The process during translation in which the complementing element of a building block interacts, by hybridisation, with the coding element of a template, thereby facilitating synthesis of a spacer backbone mediated by the catalytic activity of a ribosome.

- 15 For example decoding may be the process during translation wherein an anticodon of a tRNA molecule hybridise to the complementary codon of a template sequence thereby facilitating the ribosome mediated formation of a covalent bond between two spacer reactive groups.

Functional entity (FE)

20

A functional entity according to the present invention comprises a functional group(s) and functional entity reactive group(s) capable of linking adjacently positioned functional groups. Functional entities are in general forming part of a first building block.

25

The functional entity may comprise any desirable number of functional entity reactive groups, for example the functional entity may comprise more at least one, such as 2, for example 3, such as 4, for example 5, such as more than 5 functional entity reactive groups.

30

Within a first building block the functional entity may be separated from the spacer by a cleavable linker or by a selectively cleavable linker. The cleavable linker or the selectively cleavable linker may be attached covalently to the functional entity, for example the cleavable linker or the selectively cleavable linker may be attached

covalently to a functional entity reactive group or to a functional group of said functional entity.

Non-limiting examples of functional entities are given in figure 25.

5

FE-AA

FE-AA designates a functional entity amino acid, i.e. an amino acid covalently linked to a functional entity.

10

Functional entity reactive groups

Functional entity reactive groups are reactive groups comprised within a functional entity.

15

Corresponding functional entity reactive groups are a pair of reactive groups of different functional entities, which are capable of forming a chemical bond linking one functional group with another functional group either directly or through a linker.

Non-limiting examples of pairs of functional entity reactive groups and chemical

20

bonds that may be formed by reaction of said groups are given in figure 29.

The functional entity reactive groups may for example be selected from the group consisting of N-carboxyanhydride (NCA), N-thiocarboxyanhydride (NTA), amine, carboxylic acid, ketone, aldehyde, hydroxyl, thiol, ester, thioester, any conjugated system of double bonds, hydrazine, N-hydroxysuccinimide ester, and epoxide.

25

Furthermore, the functional entity reactive group may be an electrophile or the functional entity reactive group may be a nucleophile or the functional entity reactive group may be a radical.

30

Functional group

- 5 A functional group is a group forming part of a templated molecule. The sequence identity of functional groups in a templated molecule is a result of the capability of the template to template the synthesis of the templated molecule.

Non-limiting examples of functional groups are given in figure 24.

10 Neighbouring

- Elements, groups, entities or residues consecutive to one another in a sequence are said to be neighbouring. In particular, spacers are neighbouring, when they are part of building blocks, which comprise complementing elements, which recognise
15 coding elements that are located in sequence on a template, when said coding elements are complemented with said complementing elements.

Accordingly, preferably every spacer may have a maximum of two neighbouring spacers.

20

Non-amino acid.

Chemical entity not capable of being incorporated into a peptide by ribosome mediated translation

25 Non-standard amino acid

A non-standard amino acid is capable of being incorporated into a peptide or peptide like structure by translation mediated by a ribosome.

- 30 A non-standard amino acid according to the present invention is any amino acid comprising an amino group and a carboxyl group separated by an α -carbon. The amino acid may for example be selected from the group consisting of, Aib, Nal, Sar,

Om, Lysine analogues DAP and DAPA or any of the amino acids described in US 5,573,905. Furthermore, non-standard amino acids may be any of the above mentioned or any standard amino acids which further comprises one or more moieties selected from the group consisting of hydroxyl, bromo, fluoro, chloro, iodo, mercapto, thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, nitro, amino, alkoxy and/or amido.

The non-standard amino acid is capable of being incorporated into a peptide or peptide like structure by translation mediated by a wt, mutant, modified or recombinant ribosome.

Nucleotides

Nucleotides according to the invention includes ribonucleotides comprising a nucleobase selected from the group consisting of adenine (A), uracil (U), guanine (G), and cytosine (C), and deoxyribonucleotide comprising a nucleobase selected from the group consisting of adenine (A), thymine (T), guanine (G), and cytosine (C).

Nucleobases are capable of associating specifically with one or more other nucleobases via hydrogen bonds. Thus it is an important feature of a nucleobase that it can only form stable hydrogen bonds with one or a few other nucleobases, but that it can not form stable hydrogen bonds with most other nucleobases usually including itself. The specific interaction of one nucleobase with another nucleobase is generally termed "base-pairing".

The base pairing results in a specific hybridisation between predetermined and complementary nucleotides. Complementary nucleotides according to the present invention are nucleotides that comprise nucleobases that are capable of base-pairing.

Of the naturally occurring nucleobases adenine (A) pairs with thymine (T) or uracil (U); and guanine (G) pairs with cytosine (C). Accordingly, e.g. a nucleotide comprising A is complementary to a nucleotide comprising either T or U, and a nucleotide comprising G is complementary to a nucleotide comprising C.

Nucleotide analogues

5 Nucleotide analogues include any nucleotide analogues capable of specific base-pairing, for example derivatives of naturally occurring nucleotides or nucleotide analogues wherein the nucleotide backbone differs from naturally occurring nucleotide backbones.

Pseudo-amino acid

10

An entity comprising a substituted amino group or/and carboxyl group separated by an α -carbon or α -amine capable of being incorporated into a peptide by ribosomes.

15

For example, a pseudo amino acid may comprise a thiol group and a carboxyl group separated by an α -carbon resulting in a thioester bond in the backbone.

Examples of pseudo-amino acids are given in figure 3.

Reactive group

20

Reactive groups are groups that are capable of reacting chemically with a corresponding reactive group after being brought into reactive contact herewith.

25

For example reactive groups according to the present invention may be selected from the group consisting of di-coumarin, carboxylic acids, di-carboxylic acid, hydroxyls, diols, thioesters, amines, primary amines, secondary amines, di-amine, halogens, isocyanate, α -haloacetyl, UDP-glucose, UDP-activated saccharides, glucosyl sulphide/sulfoxide activation system (Kahne glucosylation), N-hydroxysuccinimide ester, carboxyanhydride (5-membered ring), carboxyanhydride (6 membered ring), carboxyanhydride (7-membered ring), 2,2-diphenylthiazinanone (5-membered ring), 2,2-diphenylthiazinanone (6-membered ring), 2,2-diphenylthiazinanone (7-membered ring), sulfonic acid, diamminophosphine, epoxide, thioepoxide, thiol, aldehyde, hydroxylamine, alkyl sulfonate, alkene, di-diene,

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vinylchloride unit, styrene-unit and ethylene unit. However, any other suitable reactive group, which does not impair or destroy the template may also be employed with the present invention. Examples of pairs of reactive groups are given in figure 29.

5 Recognition group

A recognition group may be part of a coding element or a complementing element. Recognition groups are involved in the recognition of a complementing element capable of recognising a coding element or in the recognition of a coding element
10 capable of recognising a complementing element. Preferred recognition groups are natural and non-natural nitrogenous bases of a naturally occurring or non-natural nucleotide.

Ribosome

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A ribosome according to the present invention is any ribosome capable of catalysing a reaction forming a covalent coupling of two amino acids selected from the group consisting of standard amino acids, non-standard amino acids, pseudo-amino acids and precursors thereof.

20

Preferably, the reaction is templated by an mRNA, to which two tRNAs, each carrying one of said standard amino acids, non-standard amino acids, pseudo-amino acids or precursors thereof. In one embodiment the covalent coupling is formation of an amide bond or an ester- or thioester bond. However, the bond could
25 be any bond formed by the reaction between a carbonyl and a nucleophile. The catalysis is dependent on the interaction between template coding elements and the complementing elements of building blocks.

30

In one embodiment of the invention, ribosomes are capable of catalysing the reaction between amino acid residues, whereby a peptide is formed.

The ribosome responsible for the incorporation of spacers according to the present invention may be any useful ribosome known to the person skilled in the art. For example the ribosome may be a wild type ribosome or a mutant, recombinant or

otherwise modified ribosome obtained from or produced by one or more suitable organism(s). One or more components of the ribosome may be synthesised in vitro by any suitable procedure such as solid-phase protein or nucleic acid chemistry.

5 Scaffold

A scaffold is a moiety comprising at least 3 reactive groups capable of reacting with functional entity reactive groups, thereby forming covalent bonds between said scaffold and said functional entities.

10

In one embodiment the scaffold is a functional entity incorporated into the spacer backbone by ribosome mediated translation. In a second embodiment of this invention the scaffold is attached covalently or non-covalently to any part of the template complexes that is not the spacer backbone. In some cases it may be beneficial to have the scaffold located externally such as on a solid support, Or the scaffold may be located in solution Furthermore, the scaffold may comprise one or more cleavable linkers, and /or the scaffold may be attached to the template or complementing template through a cleavable linker.

20 Selectively cleavable linker

Selectively cleavable linkers are not cleavable under conditions wherein a cleavable linker is cleaved. Accordingly, it is possible to cleave the cleavable linkers separating complementing entities and functional entities in a templated molecule without at the same time cleaving selectively cleavable linkers separating - in the same templated molecule - a subset of complementing entities and functional entities. It is thus possible to obtain a complex comprising a templated molecule and the template that has directed the templated- synthesis of the templated molecule, wherein the template and the templated molecule are linked by one or more, preferably one, selectively cleavable linker(s).

30

Non-limiting examples of cleavable linkers are given in figure 10. Selectively cleavable linkers should be selected according to the cleavable linkers of the specific embodiment of the invention. For example if the cleavable linkers are

cleavable by alkali treatment, the selectively cleavable linkers may for example be selected from the group consisting of linkers cleavable by photocleavage, acid cleavage, catalytic cleavage enzymatic cleavage and temperature cleavage.

5 Spacer

A spacer is a group forming part of a building block. A spacer according to the present invention comprises at least one spacer reactive group.

10 Spacers which are part of a first building block are separating the functional entity and the complementing entity of said first building block. The spacer may be covalently attached to the complementing entity of said first building block. The spacer may also be covalently attached to the functional entity of said building block, however, preferably the spacer is attached to the functional entity via a cleavable or
15 selectively cleavable linker.

Spacers which are part of second building blocks may be covalently attached to the complementing entity of said second building block.

20 In one embodiment of the present invention the spacer may be selected from the group consisting of amino acids, for example the spacer may be an α -amino acid or the spacer may be selected from the group consisting of standard amino acids, non-standard amino acids, pseudo-amino acids and derivatives thereof.

25 In one embodiment of the invention the spacer consists of a standard amino acid residue including the entire side-chain and preferably, the spacer does not form part of the templated molecule. Accordingly, said standard amino acid residue may form part of the spacer backbone, but preferably does not form part of the templated molecule except for the cases in which the standard amino acid comprise a
30 selectively cleavable linker of the templated molecule connecting the templated molecule and a spacer backbone unit.

Each spacer comprises at least one spacer reactive group, however a spacer may also comprise at least 2, such as 2, for example 3, such as more than 3 spacer reactive groups. Preferably, each spacer comprises at least 2 spacer reactive groups.

- 5 In one preferred embodiment one or more spacer reactive groups may be selected from the group consisting of acyls and amines. Preferably at least one spacer reactive group is selected from the group consisting of acyl and at least one spacer reactive group is selected from the group consisting of amines. Accordingly,
- 10 it is preferred that each spacer comprises one spacer reactive group, which is an acyl and another spacer reactive group which is an amine.

Spacer backbone

- 15 A spacer backbone according to the present invention is preferably formed by linking, by means of a reaction involving spacer reactive groups, neighbouring building block spacers. Hence, a preferred spacer backbone according to the present invention comprises, or more preferably consists of at least 3 spacer residues.
- 20 Neighbouring building block spacers are linked by a ribosome mediated reaction involving spacer reactive groups. Preferably, the reaction involves a direct chemical reaction between spacer reactive groups of neighbouring building blocks that results in the formation of a chemical bond between said two neighbouring spacers.
- 25 The chemical bond that links two neighbouring spacers may be of any suitable kind, for example the bond may be selected from the group consisting of amide bonds, aryl, acyl, ester and, thioester bonds. Examples of reactive groups are given in figure 3.
- 30 In one preferred embodiment of the present invention linking neighbouring building block spacers consists of the formation of an amide-bond. In particular, this is relevant in embodiments wherein at least one spacer reactive group of one spacer is an acyl and at least one spacer reactive group of the neighbouring spacer is an amine.

The spacer residues comprised within a spacer backbone according to the invention may be directly attached to a functional entity. Preferably, a spacer backbone according to the present invention comprises at least 3 spacers that are directly attached to a functional entity.

In one embodiment of the present invention the spacer backbone only comprises spacer residues that are directly attached to a functional entity.

In another embodiment of the present invention the spacer backbone comprises spacer residues that are directly attached to a functional entity, wherein every two spacer residues that are directly attached to a functional entity are separated by a minimum of 0 spacer residues that are not directly attached to a functional entity, for example at least 1, such as at least 2 first, for example around 2, such as around 3, for example around 4, such as around 5, for example around 6, for example around 7, such as around 8 to 10, for example around 10 to 15, such as around 15 to 20, for example around 20 to 30 spacer residues, that are not directly attached to a functional entity.

In general, the spacer backbone may be a linear sequence of spacers.

In some embodiments of the present invention it is desirable that the spacer backbone adopts a predetermined and relatively stable 3 dimensional shape after formation. This could happen gradually during synthesis of the spacer backbone or it could happen after synthesis of the entire spacer backbone. The purpose of a predetermined 3 dimensional shape of the spacer backbone may for example be to bring two predetermined functional entities close together, so that they become adjacently positioned and a functional entity reactive group of one of said two functional entities may react with a functional entity reactive group of the other of said two functional entities.

The spacer backbone may adopt any useful predetermined 3 dimensional shape. In one example the spacer backbone may for instance have the form of an α -helix. Alternatively the spacer backbone may have the form of a coiled coil. Furthermore, the spacer backbone may for example have a form selected from the group

consisting of β -sheets, beta-turn, beta-helix, coiled coils, helix-turn helix, part of a collagen structure and zinc finger structures. In addition the spacer backbone may have a 3 dimensional shape that comprises different kinds of structures, for example one or more selected from the group consisting of an α -helix, a β -sheet, coiled coil, 5 beta-turn, beta-helix, helix-turn helix, part of a collagen structure and zinc finger structures.

In one embodiment the spacer backbone is denatured and bound to a solid surface that determines the shape of the spacer backbone. For example the spacer 10 backbone may be a denatured polypeptide or derivative thereof or a denatured polypeptide like structure. The solid surface may for example be a glass surface, a plastic surface or a mineral surface,

In one embodiment when the spacer backbone has the form of an α -helix and/or 15 comprises part(s) which have the form of an α -helix, the spacer backbone preferably comprises one functional entity per helical turn of the spacer backbone. For example the spacer backbone may comprise a spacer directly attached to a functional entity for every 4 spacer residue, such as every 7 spacer residue, such as every 11 residue etc.

20 In one embodiment of the present invention the spacer backbone may be formed by a ribosome mediated incorporation of spacers. In particular, in embodiments of the present invention, wherein spacers are selected from the group consisting of amino acids, the spacer backbone may be formed by a ribosome mediated incorporation of 25 amino acids.

The ribosome, that perform the incorporation of spacers may be any useful ribosome known to the person skilled in the art. For example the ribosome may be a 30 wild type, mutant, modified or recombinant ribosome derived from any organism but preferably from *Escherichia coli*.

Spacer reactive groups

5 Spacer reactive groups according to the present invention are reactive groups (see herein above) comprised within a spacer. In particular, corresponding spacer reactive groups when brought into reactive contact with each other are capable of forming a chemical bond linking one spacer to a neighbouring spacer.

10 In one preferred embodiment of the present invention the spacer reactive groups are selected from the group consisting of acyls and amines.

Standard amino acids/residues

15 Throughout the description and claims either the three letter code or the one letter code for standard amino acids are used. Where the L or D form has not been specified it is to be understood that the amino acid in question may have the natural L form, cf. Pure & Appl. Chem. Vol. (56(5) pp 595-624 (1984) or the D form.

20 In particular standard amino acids may be selected from the group consisting of Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg and His.

25 Standard amino acid residues include residues of any of the above mentioned amino acids, that is any of the above mentioned amino acids, which have been reacted with at least one other species, such as 2, for example 3, such as more than 3 other species.

Template

30 A template according to the present invention preferably comprises a sequence of n coding elements selected from the group consisting of first coding elements and second coding elements, wherein n is an integer of more than 2. More preferably, the template comprises at least 3 first coding elements.

n is preferably an integer of more than 1 and less than 1000, for example between 5 and 500, such as between 10 and 100, for example between 3 and 100, such as between 3 and 50.

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In one embodiment the template comprises a ratio of first coding elements to second coding elements of 50:1, such as 40:1, for example 30:1, such as 25:1, for example 20:1, such as 15:1, for example 10:1, such as 8:1, for example 6:1, such as 5:1, for example 4:1, such as 3:1, for example 2:1, such as 1:1, for example 1:2, such as 1:3, for example 1:4, such as 1:5, for example 1:6, for example 1:7, such as 1:8, for example 1:10, such as 1:15, for example 1:20, such as 1:25, for example 1:30, such as 1:40, for example 1:50. However, the template may also only comprise first coding elements and no second coding elements.

15 The template preferably comprises at least 1, for example at least 2, such as at least 3, for example at least 4, such as at least 5, for example at least 10, for example at least 15, such as at least 20, for example at least 30, such as at least 40, for example at least 50, such as at least 75, for example at least 100, such as at least 150, for example at least 200 elements first coding elements.

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Furthermore the template may comprise at least 1 for example at least 2, such as at least 3, for example at least 4, such as at least 5, for example at least 10, for example at least 15, such as at least 20, for example at least 30, such as at least 40, for example at least 50, such as at least 75, for example at least 100, such as at least 150, for example at least 200 second coding elements.

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The first and the second coding elements may be dispersed between each other on the template in any desirable order.

30 In one preferred embodiment of the present invention, the template may be nucleic acid or nucleic acid analogue. Preferably, the template may be a nucleic acid, which can be template of a ribosome mediated translation.

More preferably, the template comprises or consists of RNA or a derivative or analogue thereof. For example, the template may comprise RNA residues that are

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modified on the 2' position of the ribose moiety. In some embodiments of the invention it may be preferred that the template is capped RNA. Most preferably, the template is mRNA.

- 5 In one embodiment of the present invention the template preferably comprises at least one stop coding element. For example, no corresponding complementing element may be present that can hybridise to said stop coding element.

Templated synthesis

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Templated synthesis is the process, wherein a templated molecule comprising at least 3 covalently linked functional entities is synthesised. The identity of each of the functional entities that the templated molecule comprises is defined by a predetermined template sequence.

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- Prior to the formation of the templated molecule, the functional entity is incorporated by ribosome mediated translation of a predetermined template sequence. Formation of the spacer backbone requires decoding of the template sequence by specifically charged tRNAs and the peptidyl transferase function of the ribosome. Consequently, the translation step involving template decoding and the peptidyl transferase activity of the ribosome does *not* produce the templated molecule per se but is required for choosing the functional entities that are to be assembled into the templated molecule. The covalent coupling of functional entities may occur concomitantly with the translation step or after the partial or complete synthesis of the spacer backbone in a reaction step(s) that is separate from the peptidyl transferase reaction step of the ribosome. Finally one or more of the linkages connecting one or more functional entities with the spacer backbone may be cleaved.
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- 25

- Accordingly, the templated incorporation of functional entities and their coupling to each other can take place without change in conditions, or addition of further reagents or catalysts, to the system, i.e. it can take place in a closed system. Consequently the process of choosing the functional entities according to the template sequence does not require further intervention once the process has been initiated.
- 30

However, the cleavage of the linker that links the functional entities and the spacer backbone may in some embodiments require the addition of further reagents, for example cleaving reagents.

5 In particular, templated synthesis involves contacting a sequence of coding elements with particular complementing elements. Accordingly, there is a predetermined one to one relationship between the identity of functional groups of the templated molecule and the sequence identity of first coding elements of the template that templated the synthesis of the templated molecule. Thus, during the
10 templated synthesis of the templated molecule, a functional group is initially contacting - by means of a spacer and/or a complementing element, or otherwise - the coding element capable of templating that particular functional group into the templated molecule. An oligonucleotide templated synthesis is based on an interaction of each nucleotide with its pairing partner in the template in a one-base-
15 to-one-base pairing manner. The interaction specifies the incorporation of complementing nucleotides opposite their base pairing partners in the template. Consequently, one base, including a heterocyclic base, from each oligonucleotide strand interact when forming specific base-pairs. This base pairing specificity may be achieved through Watson-Crick hydrogen-bonding interactions between the
20 bases, where the bases may be natural (i.e. A, T, G, C, U), and/or non-natural bases such as those e.g. disclosed e.g. in US 6,037,120, incorporated herein by reference. Further examples of non-natural bases nucleotides are e.g. PNA (peptide nucleic acid), LNA (locked nucleic acid) and morpholinos. Base pairing of oligonucleotides containing non-standard base pairs can be achieved by other
25 means than hydrogen bonding (e.g. interaction between hydrophobic nucleobases with "complementary" structures; Berger et al., 2000, Nucleic Acids Research, 28, pp. 2911-2914). The interacting oligonucleotide strands as well as the individual nucleotides are said to be complementary. The specificity of the interaction between oligomers results from the specific base pairing of a nucleotide with
30 another nucleotide or a predetermined subset of nucleotides, for example A base pairing with U, and C base pairing with G.

Templated molecule

5 A templated molecule within the scope of the present invention is a molecule comprising a plurality of covalently linked functional groups, wherein the templated molecule is obtainable by templated synthesis using the template.

10 In one embodiment of the present invention the templated molecule preferably comprises or essentially consists of amino acids selected from the group consisting of α -amino acids, β -amino acids, γ -amino acids, ω -amino acids.

For example, the templated molecule may comprise or essentially consist of α -amino acids, such as for example non-substituted, monosubstituted or
15 disubstituted α amino acids.

In another example the templated molecule may comprise or essentially consist of monosubstituted β -amino acids, disubstituted β -amino acids, trisubstituted β -amino acids and/or tetrasubstituted β -amino acids. In one embodiment the backbone
20 structure of said β -amino acids may comprise or essentially consist of a cyclohexane-backbone and/or a cyclopentane-backbone.

In yet another example the templated molecule may comprise or essentially consist of γ -amino acids.
25

In a still further example the templated molecule may comprise or essentially consist of ω -amino acids.

Furthermore, the templated molecule may for example comprise or essentially
30 consist of vinylogous amino acids or the templated molecule may for example comprise or essentially consist of N-substituted glycines.

Accordingly, the templated molecule according to the present invention may comprise any of a variety of different subunits. For example the templated molecule may comprise or essentially consist of functional groups and/or functional entities selected from the group of α -peptides, β -peptides, γ -peptides, ω -peptides, mono-, di- and tri-substituted α -peptides, β -peptides, γ -peptides, ω -peptides, peptides wherein the amino acid residues are in the L-form or in the D-form, vinyllogous polypeptides, glycopoly-peptides, polyamides, vinyllogous sulfonamide peptide, polysulfonamide, conjugated peptides comprising e.g. prosthetic groups, polyesters, polysaccharides, polycarbonates, polyureas, polypeptidylphosphonates, polyurethanes, azatides, oligo N-substituted glycines, polyethers, ethoxyformacetal oligomers, poly-thioethers, polyethylene glycols (PEG), polyethylenes, polydisulfides, polyarylene sulfides, polynucleotides, PNAs, LNAs, morpholinos, oligo pyrrolinone, polyoximes, polyimines, polyethyleneimines, polyimides, polyacetals, polyacetates, polystyrenes, polyvinyl, lipids, phospholipids, glycolipids, polycyclic compounds comprising e.g. aliphatic or aromatic cycles, including polyheterocyclic compounds, proteoglycans, and polysiloxanes, including any combination thereof.

Adjacently positioned residues within a templated molecule according to the present invention may be linked by any useful chemical bond, for example adjacently positioned residues of the templated molecule may be linked by a chemical bond selected from the group of chemical bonds consisting of peptide bonds, sulfonamide bonds, ester bonds, saccharide bonds, carbamate bonds, carbonate bonds, urea bonds, phosphonate bonds, urethane bonds, azatide bonds, peptoid bonds, ether bonds, ethoxy bonds, thioether bonds, single carbon bonds, double carbon bonds, triple carbon bonds, disulfide bonds, sulfide bonds, phosphodiester bonds, oxime bonds, imine bonds, imide bonds, including any combination thereof.

The backbone structure of the templated molecule will depend on the nature of the functional groups comprised within said templated molecule as well as the chemical bonds connecting functional groups of a templated molecule. Accordingly, the backbone structure of said templated molecule may for example comprise or essentially consist of a molecular group selected from $-\text{NHN}(\text{R})\text{CO}-$; $-\text{NHB}(\text{R})\text{CO}-$; $-\text{NHC}(\text{RR}')\text{CO}-$; $-\text{NHC}(=\text{CHR})\text{CO}-$; $-\text{NHC}_6\text{H}_4\text{CO}-$; $-\text{NHCH}_2\text{CHRCO}-$; $-\text{NHCHRCH}_2$

CO- ; -COCH₂- ; -COS- ; -CONR- ; -COO- ; -CSNH- ; -CH₂ NH- ; -CH₂CH₂- ; -CH₂ S- ; -CH₂ SO- ; -CH₂SO₂- ; -CH(CH₃)S- ; -CH=CH- ; -NHCO- ; -NHCONH- ; -CONHO- ; -C(=CH₂)CH₂- ; -PO₂⁻NH- ; -PO₂⁻CH₂- ; -PO₂⁻CH₂N⁺- ; -SO₂NH- ; and lactams.

- 5 A templated molecule according to the present invention may comprise any desirable number of functional groups. The functional groups of a templated molecule may all be identical, however it is also contained within the present invention that the templated molecule comprises different functional groups. For example, the templated molecule according to the invention may comprise or
- 10 essentially consist of at least 2 different functional groups, such as at least 3 different functional groups, for example at least 4 different functional groups, such as at least 5 different functional groups, for example at least 6 different functional groups, such as at least 7 different functional groups, for example at least 8 different functional groups, such as at least 9 different functional groups, for example at least
- 15 10 different functional groups, such as more than 10 different functional groups.

Template/templated molecule complex

- 20 A template/templated molecule complex is a complex comprising two parts, wherein one part templates the synthesis of the other part. Hence, one part may be synthesised by templated synthesis of the other part.

tRNA and tRNA like structures

- 25 The term tRNA according to the present invention includes any naturally occurring transfer RNA. tRNA like structures includes any molecule capable of performing the function of a tRNA, i.e. bringing a standard and/or non-standard and/or pseudo amino acid into contact with a template and thereby enabling ribosome mediated incorporation of said amino acid, non-standard- or pseudo-amino acid.

Detailed description of the invention

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Synthesis of templated molecules

Herein above are a number of prior art procedures for synthesis and functional selection of peptides, for example peptide display and PROfusion. However, in all practical terms, the described procedures are all limited to the synthesis and functional selection of α -peptides.

10

The present invention is not restricted by the above-mentioned limitations and may be employed for the synthesis of templated molecules of diverse backbone and residue chemistry.

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The present invention describes methods for synthesizing templated molecules and/or complexes and methods for targeting such molecules and/or complexes to a target species. Furthermore, the invention describes methods for amplification of templated molecules by amplification of templates that specifies selected templated molecules.

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The templated molecules according to the present invention may be specified by a template comprising n coding elements, selected from the group consisting of first coding elements and second coding elements, wherein n is an integer of more than 1, such as 2, for example 3, such as 4, for example 5, such as 6, for example 7, such as 8, for example 9, such as 10, for example in the range from 10 to 15, such as in the range from 15 to 20, for example in the range from 20 to 30, such as in the range from 30 to 40, for example in the range from 40 to 50, such as in the range from 50 to 100, for example in the range from 100 to 250, such as in the range from 250 to 500, for example n may be an integer larger than 500. In the present invention the template that encode said templated molecule is physically attached either covalently or non-covalently to the templated molecules by a technique(s)

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known to those persons skilled in the art (Peptide display, PROFusion or other means).

5 Hence, in one embodiment of the invention the template is tethered to a molecular entity capable of forming a covalent bond to the templated molecule. For example said molecular entity may be puromycin.

10 The templated molecules are preferably synthesised from second building blocks comprising a functional entity comprising a functional group and one or more functional entity reactive group(s) capable of reacting chemically with other functional entity reactive group(s) to form covalent bonds, linking functional groups of adjacent functional entities and thereby forming a templated molecule. The functional entity of a second building block may be separated from a complementing element of said building block and a spacer of said building block by a cleavable
15 linker, or a selectively cleavable linker. The complementing element is preferably capable of complementing a predetermined coding element of the template and is preferably specific for said coding element.

20 Following complementation of a coding element by a complementing element, each complementing element will present an appended group capable of being linked by spacer reactive group(s) to a neighbouring spacer presented by a neighbouring complementing element. Consecutive reactions of appended spacer of complementing elements involving spacer reactive groups of neighbouring complementing elements produce a spacer backbone exposing functional entities
25 separated from said spacer backbone by said cleavable or selectively cleavable linker(s).

30 Subsequent to spacer backbone synthesis adjacently positioned reactive groups of adjacently positioned functional entities are reacted to form a polymer or a branched molecule connected to the spacer backbone by functional groups and said cleavable or selectively cleavable linkers.

35 Cleavable linkers are cleavable under conditions wherein a selectively cleavable linker is not cleavable. Accordingly, it is possible to cleave the cleavable linkers linking the spacer backbone and functional groups in a templated molecule without

at the same time cleaving selectively cleavable linkers. It is thus possible to obtain a complex comprising a templated molecule and the spacer backbone still attached to the template that directed the synthesis of the templated molecule wherein the templated molecule and the spacer backbone are linked by one or more, preferably one, selectively cleavable linker(s).

In a further aspect it may be desirable to cleave;

- none of the cleavable linkers or,
- to cleave only a subset of cleavable linkers.

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As each complementing element is capable of recognising a predetermined coding element of a template, and as each coding element in turn defines a predetermined functional group, the sequence of coding elements of the template will template the synthesis of the templated molecule comprising a predetermined plurality of covalently linked functional groups.

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Following formation of the templated molecules, the molecules of desired properties are isolated by a selection procedure screening for relevant molecular characteristics.

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The generation of additional templated molecules can be directed from the template appended each templated molecule without any need for sequencing or any other form of characterisation. Accordingly, the complexes of the invention comprising a templated molecule linked to the template that specifies said molecule enable rapid selection and amplification of templated molecules with relevant properties.

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In a first aspect, the present invention provides a method for synthesising a templated molecule comprising a plurality of functional groups, said method comprising the steps of

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- i) providing at least one template comprising a sequence of n coding elements selected from the group consisting of first coding elements and second coding elements,

wherein each coding element comprises at least one recognition group capable of recognising a predetermined complementing element, and

wherein n is an integer of at least 3,

with the proviso that the template comprises at least 3 first coding elements,

- ii) providing a plurality of building blocks selected from the group consisting of first building blocks and second building blocks, with the proviso that at least 3 first building blocks are provided,

wherein each first building block comprises

a) at least one complementing entity comprising a first complementing element comprising at least one recognition group capable of recognising a predetermined first coding element,

b) at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and

c) at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity, and

wherein each second building block comprises

a) at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,

b) and at least one spacer comprising at least one spacer reactive group,

- 5 iii) complementing coding elements by contacting each coding element with a building block complementing element capable of recognising said coding element, wherein at least 2 coding elements are interacting with complementing elements simultaneously,
- with the proviso that a total of at least 3 first coding elements are complemented; and
- 10 iv) forming a spacer backbone by linking neighbouring spacers in a ribosome catalysed reaction by means of reacting spacer reactive groups, and
- 15 v) obtaining a templated molecule comprising at least 3 covalently linked, functional groups by linking, by means of reactioning functional entity reactive groups, a functional group of one functional entity to a functional group of another adjacently positioned, functional entity and linking said other functional entity to yet another adjacently positioned functional entity.
- 20 In a preferred aspect of the invention the templated molecule comprises a sequence of at least 3 covalently linked functional groups.
- It is comprised within the present invention that the method comprises more step(s) in addition to the above mentioned steps. In addition each step may comprise a
- 25 number of sub-steps, not explicitly mentioned herein above.
- For example, in one embodiment of the present invention step iii) to iv) of the above mentioned method comprises the steps of
- 30 a) complementing 2 neighbouring coding elements simultaneously by contacting each coding element with a building block complementing element capable of recognising said coding element, and
- 35 b) forming a spacer backbone by linking, by means of a reaction involving spacer reactive groups, the 2 building block spacers, and

c) complementing at least one further neighbouring predetermined coding element by contacting said coding element with a building block complementing element capable of recognising said coding element, and

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d) elongating the spacer backbone by linking to the spacer backbone, by means of a reaction involving spacer reactive groups, the neighbouring building block spacer.

10 The steps of the above mentioned methods may be performed in any given timely order. For example, all coding elements may be complemented before reacting neighbouring spacer reactive groups to form a spacer backbone. Alternatively, only two, such as 3, for example 4, such as 5 coding elements may be complemented before reacting neighbouring spacer reactive groups to form a spacer backbone.

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It is furthermore comprised within the present invention that the templated molecule may be formed by linking adjacent functional groups, subsequent to formation of the entire spacer backbone. Alternatively, however as soon as the spacer backbone comprises spacers of 2, such as 3, for example 4, such as 5 first building blocks, then the functional entities of said 2, such as 3, for example 4, such as 5 first building blocks may be linked to each other by reactions involving adjacent functional entity reactive groups to form a templated molecule. Subsequently, the spacer backbone may be elongated by addition of further first building block spacers and the templated molecule may accordingly be elongated, by adding functional entities of said first building blocks to the templated molecule.

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In one preferred embodiment the steps of the methods are performed chronologically in the mentioned order.

30 The individual steps of the methods may be performed any number of desirable times. For example, steps c) and d) may be repeated at least twice, such as repeated at least three times, for example at least 4 times, such as at least 5 times, for example at least 10 times, for example at least 15 times, such as at least 20 times, for example at least 30 times, such as at least 40, for example at least 50, such as at least 75 times, for example at least 100 times, such as at least 150 times,

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for example at least 200 times. Alternatively, steps c) and d) may be repeated between 2 and preferably 10,000 times, for example between 5 and preferably 1000 times, such as between 10 and preferably 500 times.

5 In addition to the above mentioned steps, it is frequently desirable to break one or more chemical bonds, for example to liberate the templated molecule from the spacer backbone or to liberate the spacer backbone from the template or to liberate the spacer backbone from individual complementing entities.

10 In one embodiment the method furthermore comprises the step of

vi) breaking the covalent bond between the spacer backbone and at least one complementing element.

15 Preferably, step vi) is performed once after every performance of step iv) or once after every performance of step b) or d) of the above mentioned methods.

Accordingly, it is preferred that the covalent bond between the spacer backbone and every complementing element is broken, hence the spacer backbone is preferably
20 not coupled covalently to any complementing element.

In another embodiment the methods furthermore comprise the step of

vii) breaking the covalent bond between the spacer backbone and at least one functional group.

25

For example 1, such as 2, for example 3, such as 4, for example 5, such as more than 5, for example more than 10, such as more than 20 covalent bonds between the spacer backbone and functional groups may be broken.

30 Said covalent bond between the spacer backbone and at least one functional group may in preferred embodiments of the invention be selected from the group consisting of cleavable linkers and selectively cleavable linkers.

Preferably, all covalent bonds between the spacer backbone and the functional
35 entities are broken except for one. More preferably, said bonds are broken after the

formation of the templated molecule. Accordingly, the spacer backbone and the templated molecule are preferably only connected to each other via one covalent bond. For example, said one covalent bond may be a selectively cleavable linker.

5 Ribosomes

Ribosomes are capable of catalysing a reaction between spacer reactive groups and thereby forming a covalent bond between spacers.

10 Preferably, ribosomes are capable of catalysing a reaction between building blocks according to the invention, such as a reaction between first building block and/or second building blocks, wherein said reaction result in the formation of a covalent bond connecting said building blocks. Preferably, said covalent bond is formed between spacers of the individual building blocks.

15 A preferred ribosome according to the invention is capable of catalysing a reaction between t-RNAs charged with spacers, whereby a covalent bond is formed between the spacers of two charged t-RNAs. Even more preferably, a ribosome according to the present invention is capable of catalysing a reaction between t-RNAs charged
20 with a spacer selected from the group consisting of FE-AA, standard amino acids, non-standard amino acids, pseudo-amino acids and precursors thereof, whereby a covalent bond is formed between said spacers.

25 Most preferably, a ribosome is capable of catalysing a reaction between t-RNAs charged with standard amino acids, whereby a peptide is formed.

30 The ribosome may be a wild type ribosome derived from for example an animal, for example a mammal, such as a human being, a plant, a fungi, a yeast or a bacterium. In addition a ribosome may be a mutant or a recombinantly modified version of a wild type ribosome. Methods of genetically engineering a ribosome is known to the person skilled in the art.

Preferably, such a mutant or recombinantly modified ribosome comprises essentially the same activity as a wild type ribosome, such as at least 60%, for example at least

70%, such as at least 80%, for example at least 90%, such as at least 95%, for example at least 99% of the activity of a wild type ribosome. Said activity may be determined by assay measuring the rate of synthesis of a polypeptide.

- 5 The ribosome may be a wild type ribosome purified from any organism for example an animal, for example a mammal, such as a human being, a plant, a fungi, a yeast or a bacterium. Alternatively, the ribosome may have been produced using recombinant technology.

10 Templated molecules

- 15 In another aspect, the present invention relates to a templated molecule, a plurality of the same or different templated molecules, wherein preferably each of the templated molecules are obtainable by a method for synthesizing templated molecules according to the present invention.

- 20 For example the invention relates to a templated molecule covalently linked to the template encoding said templated molecule, wherein said templated molecule comprises at least 3 covalently linked, functional groups, each encoded by a coding element of said template, with the proviso, that the templated molecule is not a standard α -polypeptide.

- 25 Furthermore, the invention relates to a plurality of templated molecules, wherein the plurality comprises at least 1000, such as in the range from 1000 to 5000, for example in the range from 5000 to 10,000, such as in the range from 10,000 to 50,000, for example in the range from 50,000 to 100,000 such as in the range from 100,000 to 500,000, for example in the range from 500,000 to 1,000,000 such as in the range from 1,000,000 to 5,000,000, for example in the range from 5,000,000 to 10,000,000 different templated molecules and wherein said templated molecule
30 comprises at least 3 covalently linked, functional groups, each encoded by a coding element of a template, with the proviso, that the templated molecule is not a standard α -polypeptide.

In particular, the plurality of templated molecules may comprise templated molecules, wherein each templated molecule is selected from the group consisting of templated molecules covalently linked to the template encoding said templated molecule.

5

The amplifiability of the templated molecules in a molecule library provides said library with a unique feature. This unique feature involves e.g. that a vast number of templated molecules can be screened by taking the library through repetitive processes of selection-and-amplification, in a parallel process where the library of molecules is treated as a whole, and where it is not necessary to characterise individual molecules (or even the population of molecules) between selection-and-amplification rounds.

It is possible according to various preferred embodiments of the invention to screen e.g. more than or about 10^3 different templated molecules, such as more than or about 10^4 different templated molecules, for example more than or about 10^5 different templated molecules, such as more than or about 10^6 different templated molecules, for example more than or about 10^7 different templated molecules, such as more than or about 10^8 different templated molecules, for example more than or about 10^9 different templated molecules, such as more than or about 10^{10} different templated molecules, for example more than or about 10^{11} different templated molecules, such as more than or about 10^{12} different templated molecules, for example more than or about 10^{13} different templated molecules, such as more than or about 10^{14} different templated molecules, for example more than or about 10^{15} different templated molecules, such as more than or about 10^{16} different templated molecules, for example more than or about 10^{17} different templated molecules, such as more than or about 10^{18} different templated molecules.

As one may perform many repetitive rounds of parallel selection and parallel amplification processes, it is possible to enrich only e.g. 100 fold in each round, and still get a very efficient enrichment, of e.g. 10^{14} fold over a number of selection-and-amplification rounds (theoretically a 10^{14} fold enrichment is obtained after seven rounds each enriching 100 fold). To obtain a similar enrichment of 10^{14} fold using a non-amplifiable library, would require screening conditions allowing 10^{14} fold enrichment in one "round" - and this is not practically possible using state-of-the-art

screening technologies. The templated molecules and/or the templates can furthermore be bound to a solid or semi-solid support.

Methods for screening

5

In even further aspects the methods of the invention - individually or as a combination - relates to

10 a method for screening a composition of complexes or templated molecules potentially having a predetermined activity,

a method for assaying the predetermined activity potentially associated with the complexes or the templated molecules,

15 a method for selecting complexes or templated molecules having a predetermined activity,

20 a method for amplification of the template that templated the synthesis of the templated molecule having, or potentially having a predetermined activity, and

a method for amplification of the template that templated the synthesis of the templated molecule having, or potentially having, a predetermined activity, said method comprising the further step of increasing the number of copies of the templated molecule.

25

Preferred embodiments of the invention

In a preferred embodiment of this invention a first building blocks comprising:

30 a) at least one complementing entity comprising at least one recognition group capable of recognising a predetermined coding.

b) at least one functional entity comprising at least one functional group and at least one functional entity reactive group

c) at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity

5

is a tRNA or "tRNA-like" molecule specifying said complementing entities, said functional entities, said spacers and said spacer reactive groups.

In a preferred aspect of this invention a subset of building blocks each comprising

10

a) at least one complementing entity comprising at least one recognition group capable of recognising a predetermined coding element, and

b) at least one spacer comprising at least one spacer reactive group.

15

are tRNA or "tRNA-like" molecules specifying said complementing entities, said spacer and said spacer reactive groups.

In a preferred aspect of this invention the linking of spacer groups are performed by ribosomes or by other entities (e.g. DNA, RNA, proteins or combinations thereof, such as ribozymes) or combination of entities (such as ribozyme/protein complexes) enabling template dependent formation of a covalent bond, but preferably by ribosomes.

20

In a preferred embodiment of this invention the template (or coding element) is DNA, RNA or modified versions thereof including phosphorothiate DNA -or RNA, 2'-O-methyl RNA or mixed nucleic acid sequences, but preferably RNA.

25

In a preferred aspect of this invention the template that specified the templated molecule is linked to the templated molecule by any covalent or non-covalent means linking the templated molecule and the template. Such linkages may be accomplished by techniques known to those skilled in the art, and may include SPERT or PROFusion (described above) or equivalent techniques.

30

In one preferred embodiment, the templated molecule is linked to its template by any of the linkages or methods described in patents US 6,214,553 or US 6,207,446, which are both incorporated herein by reference in their entirety. For example, the templated molecule may be linked to its template using puromycin, for example, the
5 template may be tethered to puromycin, which may become covalently linked to the templated molecule.

Hence, in the template/templated molecule complex according to the invention, the template may be linked to the templated molecule via a puromycin linker.

10

According to the above statements the preferred embodiment of this invention relates to;

a) translation of RNA or other nucleic acid- or nucleic acid derivative templates by
15 ribosomes or by other entities (e.g. DNA, RNA, proteins or combinations thereof) or combination of entities enabling template-specific formation of covalent bond(s).

b) template complementation involving tRNAs or tRNA-like entities comprising a template complementing element, preferably an anticodon, enabling
20 complementation of a template element, preferably a codon.

c) tRNAs or tRNA-like entities comprising a spacer reactive group or groups enabling covalent coupling between a neighbouring spacers or spacers provided by a neighbouring tRNA or tRNA-like entity or entities according to the interaction to the
25 template.

d) formation of a spacer backbone by formation of a plurality of reactions between spacer reactive groups, a reaction preferably catalysed by a ribosome.

e) tRNAs or tRNA-like entities covalently linked to a spacer reactive group(s) wherein the spacer reactive groups are preferably an acyl and/or an amine.

f) tRNAs and tRNA-like entities where the spacer reactive groups are part of a standard α -amino acid, non-standard amino acid, pseudo-amino acid.

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- g) tRNAs and tRNA-like entities where a subset of tRNA and tRNA-like entities comprise a functional entity (FE) comprising a functional group and functional entity reactive groups capable of forming a covalent link to a neighbouring functional reactive group(s). Each functional group is linked by a cleavable or selectively cleavable linker to the spacer reactive groups. Preferably the functional entity and the cleavable or selectively cleavable linker separating the functional entity and complementing element is an R-side-group of a *i*) standard α -amino acid, *ii*) non-standard amino acid or *iii*) pseudo-amino acid.
- h) formation of a spacer backbone which is preferably a standard α -peptide backbone, non-standard- or pseudo-peptide backbone.
- i) formation of a spacer backbone comprising two or more functional entities each comprising one or more functional entity reactive group(s) capable of linking by reaction to one or more neighbouring functional entity reactive group(s).
- j) coupling of neighbouring functional reactive groups and the formation of a templated molecule of linked functional groups with each functional group linked to at least one neighbouring functional groups and each functional group further linked to the spacer backbone through the cleavable or selectively linkers.
- k) cleavage of none, one or more of the of cleavable linkers, leaving one or more, preferably one, selectively cleavable linker intact, enabling preferably a single covalent coupling between the assembled functionalities of functionalities and the spacer backbone.
- l) formation of a covalent or non-covalent link between the templated molecule and the template that templated the templated molecule, preferably the link is via the spacer backbone.
- Accordingly, a preferred aspect of this invention relates to the incorporation in the process of translation of non-standard amino acids, comprising or linked to functional entities comprising functional entity reactive group(s), into a peptide comprising an amide- or other type of bonds, but preferably an α -peptide. The non-standard or pseudo amino acids comprising or linked to said functional entities,

hereafter termed FE-AA (functional entity-amino acid) are incorporated into a peptide by ribosomes using tRNA or tRNA-like entities for template decoding where the template preferably constitute an RNA sequence or a modified RNA sequence containing at least one translational start site, preferably AUG and a coding sequence capable of being decoded by said tRNAs or tRNA-like entities.

A FE-AA is attached to a specific tRNA or tRNA-like entity that specifically decode a coding element or a set of coding elements in the RNA template by the interaction between the codon of the RNA template and the anticodon of said tRNA or tRNA-like entity during translation. Preferably, the FE-AA is attached to its cognate tRNA or tRNA-like entity by an acyl-linker similar to that observed for charging of tRNAs by standard amino acids for the purpose of translation. An example of a tRNA charged with an amino acid and the corresponding charging with an FE-AA is shown in figure 4B and 4A, respectively. A few examples of tRNAs charged with FE-AA units are shown in figure 4C. Since individual tRNAs or tRNA-like entities are selectively charged with individual specific FE-AA units and is capable of decoding a specific codon of the template it is possible to incorporate a plethora of FE-AA units into a peptide sequence at predetermined positions according to the template sequence.

Charging of individual tRNAs with cognate FE-AA units or when desired with standard-, non-standard or pseudo-amino acids can be accomplished by at least two protocols known to those persons skilled in the art;

i) Enzymatic charging of tRNAs using wt or engineered versions of amino acyl-tRNA synthetases capable of linking by formation of a covalent bond, a specific tRNA and a specific FE-AA unit, standard-, non-standard- or pseudo-amino acid. The linking may be any covalent bond, but preferably an acyl bond between a 2' or 3' OH-group of the tRNA 3'-terminal adenosine nucleotide and a carboxyl group of said, FE-AA unit, standard-, non-standard or pseudo-amino-acids. See figure 5A

ii) Chemical aminoacylation of tRNAs. Here, each specific in vitro synthesised tRNA missing the 3'-terminal pCpA dinucleotide is ligated by an enzymatic reaction to a pdCpA-dinucleotide chemically charged with a FE-AA unit, standard-, non-standard or pseudo-amino acid. The linking may be any covalent bond, but preferably an acyl bond between a 2' or 3' OH-group of the tRNA 3'-terminal nucleotide and a carboxyl

group (or activated ester) of said, FE-AA unit, standard-, non-standard or pseudo-amino acid. See Figure 5B.

- Each peptide should contain atleast 2 FE-AA for example 3 FE-AA, such as atleast 4 FE-AA, for example 5 FE-AA, such as atleast 8 FE-AA, for example 10 FE-AA, such as atleast 15 FE-AA, for example 20 FE-AA, such as 30 FE-AA, for example 50 FE-AA, such as 100 FE-AA, for example 200 FE-AA, such as atleast 300 FE-AA for example 500 FE-AA. It may be desirable that a peptide contain standard, non-standard or pseudo-aminoacids that does not constitute an FE-AA unit. Such non-FE-AA units are preferably not a part of a templated molecule but may serve structural purposes necessary for ordered polymerisation/bond formation required for the formation of the templated molecule according to the predetermined template sequence as described below.
- Evidently, a preferred embodiment of this invention dictates that a translational apparatus should be capable of sustaining the conversion of a predetermined template sequence into a standard peptide, non-peptide or pseudo-peptide sequence comprising sidegroups which are not confined to the 20 amino acid sidegroups found in naturally occurring peptides. Several experiments have shown using synthetically engineered tRNAs carrying non-standard amino acid units that both eukaryotic and prokaryotic translation machineries can incorporate said non-standard amino acid or pseudo amino acids in a peptide chain. Consequently, a translational machinery is capable of incorporation non-standard amino acid or pseudo-amino acids such as many L-aminoacids comprising unusual sidegroups, alpha-alpha-disubstituted aminoacids, alpha-hydroxy acids such as lactic-, glucolic- or phenyllactic acids, mercapto acids or N-methyl-aminoacids, conformationally restricted amino acids, amino acids with photoaffinity labels, spin labels and unusual steric properties etc. An incomprehensive list of molecules which have been shown to be incorporated into peptides by in vivo, in situ or in vitro translation by cells, tissues, extracts or purified components of a translational machinery of eukaryotic or prokaryotic origin as shown in figure 3. The vast chemical and structural diversity of the molecules capable of being incorporated in peptides suggests that the translation process exhibit only a limited discrimination and further argues that most alpha-aminoacids sidegroups including desirable FE sidegroups can easily be incorporated into peptides by ribosomes. The sizes and functionalities of FE-AA

units that are to be incorporated by ribosome mediated translation according to this invention are similar to the sizes and functionalities listed in figure 3.

It may be desirable that translation is conducted by purified reconstituted translation components such as PURE (Protein synthesis using recombinant elements; Shimizu et al., 2001). Translation using purified components offers several advantages;

i) Each specific tRNA capable of recognising a specific codon can be charged with any FE-AA unit, standard-, non-standard or pseudo-amino acid without limitations.

ii) tRNAs charged with FE-AA units, non-standard or pseudo-aminoacids are not deacylated by aminoacyl-tRNA synthetases.

iii) release factors (RF1, RF2 and RF3) can be omitted from the translation reaction preventing premature translation arrest by stop sites and further expands the number of codons by allowing tRNA reading of the UAG, UAA and UGA codons.

iv) reduced level of background "noise" (i.e. irrelevant protein, RNA and DNA).

v) the absence of ribonucleases will increase RNA stability and promote template recovery.

In one aspect of this invention release factors (RF1-3) are omitted from the translation reaction which allows specifically engineered tRNAs to decode the template codons UAG, UGA and UAA normally specifying a translational stop-site. This expands the codon-set and furthermore prevent premature translation-arrest as well as increases the efficiency of template-peptide coupling (when employing PROFusion or similar coupling technology). However, in some aspects of this invention, one or more release factors may be included in a translation reaction.

An FE-AA unit is incorporated into the spacer backbone according to the template sequence. However, the subsequent polymerisation of FE-units is based on a reaction between a reactive group(s) of an FE-unit with a reactive group(s) of an adjacent FE-unit and, thus, governed by a proximity effect. Consequently, in one embodiment of this invention functional groups specified by the template are

assembled into the templated molecule in random order resulting in sequences of functional groups that may not correspond to the order specified by the template sequence.

- 5 In a further aspect it may be desirable to incorporate an FE-AA unit comprising a scaffolding molecule (see f. ex Figure 4-18) for attaching functional groups producing a templated molecule comprised of the scaffolding molecule and functional groups specified by the template sequence and assembled on the scaffolding molecule at random.

10

Ultimately, the selected template sequences specifying a limited number of functional entities assembled at random allows for the synthesis of each molecule candidate individually for further examination.

- 15 In a preferred aspect of this invention the assembly of functional groups forming the templated molecule occurs in a non-random and predetermined order. In some embodiments the spacer backbone may adopt predetermined 3-dimensional structures that enable the ordered assembly of functional groups by reactions between FE-reactive groups positioned adjacently according to the 3-dimensional structure. Such structures are formed either concomitantly with or after synthesis of the spacer backbone. In a preferred aspect such structures may be specified by spacer residues that are *not* FE-units. In one aspect the spacer backbone may adopt the form of simple structures such as an α -helix, parallel or anti-parallel β -sheet or β -turn. However, other higher order structures may include helix-turn-helix and zinc-finger structures, left and right-handed di-, tri-, tetra- or penta-meric coiled-coils, cystine knots, 3_{10} -helices and parallel β -helix as well as structures obtainable by interactions with various supports such as a glass-, plastic- and mineral-surfaces, interaction with nucleic acids, solvents and ice-matrices or structure stabilization by magnetic fields. A few relevant structures and their application in the assembly of templated molecules are described below.
- 20
- 25
- 30

- α -helix: The α -helix is a coiled, mainly right-handed, structure present in nearly all natural proteins. The right-handed α -helix is stabilised by an array of intra-strand hydrogen-bonds. Some amino acids side-groups such as alanine, glutamate, leucine, isoleucine have a preference for forming an α -helix. Since the helix coil
- 35

consists of ~ 3.6 amino acid residues per helix turn the side-chains may alter between charged and hydrophobic residues with a periodicity of three or four amino acids creating surface patches with defined properties along the helical axis (Figure 7A). These properties may define helix solubility, stability or the propensity for interaction with other helices to form coiled-coil superstructures as described below. In one aspect of this invention standard, non-standard or pseudo-amino acids are incorporated into a spacer backbone at positions predetermined by the template sequence intended for α -helix formation. FE-AA units are incorporated into said spacer backbone at positions specified by the template preferably at a periodicity of three or four amino acids forming a patch of FE-units arranged at the same face of the helix specified by the spacer backbone (see Figure 7B). Thus, on average an FE-AA unit is incorporated once per helix turn resulting in distance of ~ 6 Å between neighbouring FE-AA units in the helical array. Incorporation of one FE-AA unit per two helical turns results in a distance of ~11 Å between adjacent FE-units. Incorporation of one FE-AA unit per three helical turns results in a distance of ~17 Å between adjacent FE-units. Incorporation of one FE-AA unit per four helical turns results in a distance of ~ 22 Å between adjacent FE-units etc. Yet, in a preferred aspect of this invention the FE-AA units are incorporated into the spacer backbone once per helical turn. Consequently, each FE-unit is positioned in close proximity of a neighbouring FE-unit(s) for optimal reaction between adjacent FE-reactive groups enabling efficient FE polymerization and the ordered assembly of the templated molecule according to the template sequence.

Even though some amino acid residues have the propensity for α -helix formation, the single α -helical structure is generally considered unstable and the predictability of such helical structure unreliable. In some cases it may be beneficial to attach negatively charged entities at the C-terminal and/or a positively charged entity at the N-terminal residue of the spacer backbone as dipole-compensating residues augmenting helix stability. In yet another aspect it may be advantageous to apply magnetic field for helix orientation and stabilization according to the helix dipole-moment. In another aspect a putative α -helical structure may be stabilised by solvents or by interactions with polymers such as polyethylene-glycol, poly-phosphate or poly-sialyl via interactions between negatively or positively charged patches of the helix and the counter-ions of the polymer. In a further aspect uncharged or hydrophobic residues of an α -helix may increase helix stability by

interactions with uncharged or hydrophobic units in a polymer. Furthermore, a putative α -helix may be stabilised by interaction with biomolecules such double-stranded DNA or RNA where the imidazole ring of histidine, or the charged amines of lysine and arginine arrayed into patches on the helix can facilitate non-specific interaction with DNA or RNA backbone phosphate-groups, preferably in the minor-groove. Accordingly, the template sequence could be designed such that the spacer backbone conforms to an amphipathic α -helix where FE-units constitute one face of the helix whereas the opposite face comprises residues required for α -helix stability and/or interaction with a helix organising entity. In this set-up the polymerization between FE-units arrayed along the helical axis is initiated after helix stabilization which allows directional polymerisation of FE-units according to the template sequence. Subsequent activation of the templated molecule by cleavage of one or more cleavable linkers is independent of helix stability.

In a preferred aspect of this invention the spacer backbone is an α -helix stabilised by inter- or intramolecular interaction with one or more α -helices forming coiled-coil structure(s).

Coiled-coils: Coiled coils are a bundle of α -helical coils wound into a superhelix (see Figure 8). All coiled-coils have a distinctive repetitive sequence of hydrophobic and hydrophilic residues - the heptad repeat. Each α -helix contains 3.5 residues per turn so that the positions of every seventh residue are eclipsed on the helical surface (i.e. occupy the same radial position when viewed on the helical wheel). In most natural and designed coiled-coils leucine residues constitute the hydrophobic core involved in inter-helical pairing resulting in dimers, trimers, tetramers or pentamers dependent on the level of helix hydrophobicity. Coiled-coils may be parallel or anti-parallel and contain any of several possible heptad repetitive sequences comprising interchanging hydrophobic and hydrophilic residues according to the consensus HPPHPPH, HPPPHPP or HPPHPPP (H = hydrophobic residues, P = polar residues). A subset of coiled-coils are leucine zippers comprising repetitive heptad sequences of the consensus LXXYXXX or YXXLXXX where L denotes leucine, Y is leucine, isoleucine or valine and X any amino acid. The structures may be stabilised further by oppositely charged residues positioned in register on opposing helices resulting in electrostatic interactions referred to as acid-base coiled-coils (see Figure 8). Further coiled-coil stabilising elements may include cysteine residues positioned

at helix ends forming inter-helical disulfide bonds or β -lactam units increasing core hydrophobicity.

FE-AA units can be incorporated into a spacer backbone predisposed for coiled-coil formation. Furthermore, the FE-AA units can be incorporated into a spacer backbone according to the template sequence positioned opposite the hydrophobic core involved in dimerisation and thus exposed into solution. In a preferred aspect of this invention it is possible to incorporate FE-AA units once per helical turn, once per two helical turns, once per three helical turns or once per four helical turns etc, but preferably once per helical turn within the repetitive helical segments of the coiled-coil structure. A few examples of repetitive heptad sequences designed for dimer coiled-coil structure formation are listed below.

M-C-X_n-(L-K-U-E-Y-P-U)_n-X_n-C-X
 M-C-X_n-(L-E-U-K-Y-P-U)_n-X_n-C-X
 M-C-X_n-(L-K-U-E-Y-U-P)_n-X_n-C-X
 M-C-X_n-(L-E-U-K-Y-U-P)_n-X_n-C-X

M = Methionine, C = Cysteine, P = polar residue, U = FE-unit.

Insertion of cysteines for di-sulfide bridge formation or E and K for inter-strand ionic interactions are optional but may be beneficial in cases where the FE-polymerisation reaction is conducted in adverse conditions (high temperature, high pH, in solvents etc.).

Coiled-coil formation requires that the spacer backbone comprising the FE-units should dimerise with a second helical element predisposed for coiled-coil structure formation. Thus, the spacer backbone comprising FE-units may dimerise with another spacer backbone comprising FE-units or with a helical element that does not contain FE-units.

Peptide strands that does not contain FE-units may be produced separately by solid-phase chemical synthesis

In another aspect it is possible that a spacer backbone comprising FE-units contain two helical elements each predisposed for coiled-coil formation separated by a turn in the spacer backbone structure resulting in an intra-molecular coiled-coil.

5 In yet another aspect of this invention it is possible to form trimers, tetramers or pentamers by increasing the number of hydrophobic residues. The use of higher order coiled-coil structures may be useful for the polymerisation of displayed FE-units in solvents, at high pH or at high temperatures.

10 Collagen triple-helix: Collagen is a highly stable triple helix structure where each peptide strand contains repetitive units of the sequence;

Glycin-Proline-X ,

15 where, X denotes any aminoacid, preferably a polar residue. The single backbone strand contains ~ 3 residues per turn with a helical rise of 2.9 Å. Consequently, it is possible to incorporate FE-AA units into a spacer backbone comprising the following repetitive trimeric sequence;

20 (Glycine-Proline-U)_n , U = FE-unit.

After formation of a triple helix super-structure each FE-unit will be displayed into solution. The average distance between neighbouring FE-units will be ~ 9Å.

25 Each strand displaying FE-unit may associate with one or two other strands containing FE-units or with one or two strands not containing FE-units. Peptide strands not containing FE-units may be produced separately by solid-phase chemical synthesis.

30 It may be advantageous to use a collagen triple-helix structure for FE-display in the cases where the polymerisation reaction is conducted under conditions not easily compatible with protein secondary structure.

35 β-helix: This structure forms multiple parallel sheeted folds forming a tube structure. Sheet residues either protrude into the largely hydrophobic interior of the tube or outwards into solution and adjacent residues on the same face of neighbouring

5 sheets are separated by less than 5 Å. Thus, in one aspect it is possible to incorporate FE-AA units displayed in either a hydrophobic or a polar environment. Furthermore, the close proximity of neighbouring FE-units should benefit the overall polymerisation efficiency, thus increasing the production of templated molecules.

5

In a preferred aspect of this invention the FE-display structures described above require that only a limited number of the total codon-set is used for incorporation of standard-, non-standard- or pseudo-amino acids (i. e. non-FE-units). Preferably, less than 5 codons are used for the incorporation of non-FE-units according to the invention

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Thus, the present invention disclose a method for templating a templated molecule comprising FE-units specified by the template sequence.

15

Provided that complementation of neighbouring coding elements is achieved, neighbouring, spacer reactive groups of a building block are capable of being covalently linked forming a spacer backbone. The random or non-random display of FE-units enable reactions between FE-reactive groups of neighbouring FE-units forming a polymer or a branched molecule of FE-units specified by the template sequence. In a further application it is possible subsequently to maintain or cleave the cleavable linker separating the functional entity from the complementing element defining said functional entity without cleaving the link between neighbouring functional groups of a templated molecule.

20

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Also disclosed are methods for identifying the sequence and/or identity of functional groups of a templated molecule, as well as methods for therapy and diagnostic methods exploiting the templated molecules according to the invention.

30

In yet another aspect there is provided a method for altering the sequence of a templated molecule, including generating a templated molecule comprising a novel or altered sequence of functional groups, wherein the method comprises the step of mutating the template that templated the synthesis of the original templated molecule. The method preferably comprises the steps of

providing a first template capable of templating the first templated molecule, or a plurality of such templates capable of templating a plurality of first templated molecules,

- 5 modifying the sequence of the first template, or the plurality or first templates, and generating a second template, or a plurality of second templates,

wherein said second template(s) is capable of templating the synthesis of a second templated molecule, or a plurality of second templated molecules,

10

wherein said second templated molecule(s) comprises a sequence of covalently linked, functional groups that is not identical to the sequence of functional groups of the first templated molecule(s), and optionally

- 15 templating by means of said second template(s) a second templated molecule, or a plurality of such second templated molecules.

The above-mentioned method exploits that a templated synthesis in one embodiment involves a single-stranded, modifiable intermediate in the form of a
20 template. In the case where this template comprises a nucleotide strand comprising deoxyribonucleotides or ribonucleotides, most molecular biological methods can be applied to modify the template, and therefore to modify the templated molecule.

The present invention also relates to building blocks used for synthesising the
25 templated molecule and to complexes comprising such building blocks. In another aspect there is provided the use of a building block for the synthesis of a templated molecule according to the invention. In a preferred embodiment of this aspect, the templated molecule comprises or essentially consists of a molecular entity capable of binding to another molecular entity in the form of a target molecular entity or a
30 binding partner.

The templated molecule is preferably a medicament capable of being administered in a pharmaceutically effective amount in a pharmaceutical composition to an individual and treating a clinical condition in said individual in need of such
35 treatment.

5 In other aspects of the invention there are provided a pesticidal composition, an insecticidal or herbicidal composition, a bacteriocidal or bacteriostatic composition, and a fungicidal composition, as well as methods for preparing such compositions and uses thereof, wherein each of said compositions comprise a templated molecule according to the invention in an amount effective to achieve a desired effect.

10 In still further aspects there is provided a method for identifying a pharmaceutical agent, or a diagnostic agent, wherein said method comprises the step of screening a plurality of drug targets with at least one predetermined, templated molecule, and identifying a pharmaceutical agent, or a diagnostic agent, in the form of candidate templated molecules capable of interacting with said drug targets.

15 In yet another aspect there is provided a method for identifying a target, including a drug target, wherein said method comprises the step of screening a plurality of ligands or receptor moieties with at least one predetermined, templated molecule, and identifying drug targets in the form of ligands or receptor moieties capable of interacting with said templated molecules.

20 The present invention also relates to any isolated or purified templated molecule having an affinity for a predetermined target, including a drug target, as well as to targets, including drug targets, in the form of ligands, receptor moieties, enzymes, cell surfaces, solid or semi-solid surfaces, as well as any other physical or molecular entity or surface having an affinity for a predetermined templated molecule.

25

In even further aspects of the invention there is provided a method for treatment of an individual in need thereof, said method comprises the step of administering to the individual a pharmaceutically effective amount of a molecule identified by a method

30 of the present invention and having an affinity for a predetermined target, including a drug target.

In a still further aspect there is provided a method for treatment of an individual in need thereof, said method comprises the step of administering to the individual a pharmaceutically effective amount of an isolated or purified ligand or receptor moiety

35

having an affinity for a predetermined templated molecule according to the invention. The isolated or purified ligand or receptor moiety is preferably identified by the above-mentioned method of identification of the invention.

5

Examples

The following example illustrates specific embodiments of the invention and should not be regarded as limiting for the invention.

10

Example of synthesis, selection and amplification of encoded β -peptide molecules capable of binding to a receptor protein.

Below is described the synthesis of a library of molecules capable of being tested for desired characteristics. Molecules with relevant properties can be selected and their templates amplified allowing enrichment of templates encoding said molecules. Multiple rounds of molecule selection and amplification of co-selected templates enable the isolation of molecules with unique properties.

20 A DNA template is provided encoding a T7-RNA promoter sequence, a translational initiation element comprising a Shine&Dalgarno and an ATG codon followed by a random sequence of 18 nucleotides and a fixed sequence of 16 nucleotides with the sequence; 5'-TAGTCCGAATCCCGGG-3'. Said template is transcribed producing at least 10^{13} different RNA molecules according to a standard procedure with the following composition: 100 mM Tris-HCl, 22 mM $MgCl_2$, 4 mM each of UTP, CTP, ATP, GTP, 10 mM DTT, 10^{13} DNA different template molecules, 1u/ μ l RNasin, and 1u/ μ l T7 RNA polymerase and incubated at 37°C for 4h.

25 Following RNA synthesis the DNA template is removed by addition of 10 units of DNase I. The RNA template molecules are recovered through gel-filtration or by preparative gel-electrophoresis according to standard procedures.

30 Purified RNA is tagged by DNA-puromycin entity at the 3'-end according to the method shown in figure 2 (Roberts et al.1997). In brief, a fixed DNA sequence dA27dCdC- followed by a 3'-terminal puromycin residue is attached to the RNA 3'-end by ligation using T4 RNA ligase and a DNA oligonucleotide that enhance the

ligation efficiency. Following addition of the DNA-puromycin-tag the RNA-DNA-puromycin chimeric molecules are purified by gel-filtration or preparative gel-electrophoresis.

- 5 The purified and tagged RNA fragments are in vitro translated using standard in vitro translation or preferably by purified components (PURE, Shimizu et al., 2001) and tRNAs charged with FE-AA units, standard, non-standard or pseudo-amino acids. The tRNAs to be charged are synthesised from plasmid preparations carrying a specific anticodon triplet sequence but missing the 3'-end CpA dinucleotide in a
- 10 tRNA synthesis mixture with the following composition: 100 mM Tris-HCl, 22 mM MgCl₂, 4 mM each of UTP, CTP, ATP, GTP, 10 mM DTT, 2 mM spermidine, 200 µg of DNA template molecules, 1u/µl RNasin, and 1u/µl T7 RNA polymerase in total volume of 1 ml and incubated at 37°C for 4h. The above synthesis is conducted for 64 different plasmid templates each encoding a tRNA sequence containing a unique
- 15 anticodon triplet sequence. Subsequently, the pre-tRNAs (missing the CpA dinucleotide) is purified using gel-filtration, gel-electrophoresis or preparative HPLC.

A pdCpA dinucleotide is purchased or synthesised by standard phosphoramidite chemistry described in Robertson et al., 1989, 1991.

20

- Prior to the charging of the tRNAs, pdCpA dinucleotides are chemically acylated using protected FE-AA, amino acids, non-amino acids or pseudo-amino acids. Preferably, the cyanomethyl esters of FE-AA, amino acids, non-natural amino acids or pseudo-amino acids are used for the selective mono-acylation of 3' or 2' hydroxyl
- 25 groups of the adenosine of the pdCpA dinucleotide. Protection of sensitive amino groups of FE-AA, natural-, nonnatural- and pseudo-amino acids can be accomplished by formation of their nitroveratryloxy (NVOC) carbamate, ester or ether derivatives as described elsewhere (Robertson et al., 1991). Alternatively, the biphenylisopropylloxycarbonyl (BPOC) protective group have been used (Robertson et al., 1991). These protective groups can be removed at any convenient step prior
- 30 to translation by photoactivation or mildly acid conditions, respectively (Robertson et al., 1991; Mendel et al., 1995). Here, initial amino-protection is by *t*-butyl (Boc) carbamate formed by addition of (Boc)₂ and H₂CO₃ for 30 min at room temperature. Following protection, solid calcium phosphate is added and the product extracted by
- 35 ethylacetate.

Formation of cyanomethylester derivatives of FE-AA, amino acid, non-natural- and pseudo-amino acid for the pdCpA acylation step is accomplished by standard chemistry f.ex using DCC (dicyclocarbodiimide) and hydroxyacetonitrile.

5

Coupling of the cyanomethylesters and the pdCpA dinucleotide is accomplished by reaction between the cyanomethyl ester derivative and the tetrabutyl ammonium salt of pdCpA in DMF and triethylamin at 50 °C for 2 hours (Modified from the protocol of Robertson et al., 1989, 1991). The Boc-amino protective groups is removed by addition of CCl₃COOH to the mixture and reacted for another hour. The charged and amino deprotected acylated dinucleotide is purified using preparative HPLC.

10

Each individual dinucleotide charged with a specific FE-AA, natural-, nonnatural- or pseudo-amino acid is coupled to a specific pre-tRNA using T4 RNA ligase in a reaction of the following composition: 42 mM HEPES-KOH, pH 7.4, 10 DMSO, 4 mM DTT, 20 mM MgCl₂, 0.2 mg/ml bovine serum albumin, 150 µM ATP, 10 % PEG6000, 2,000 units T4 RNA ligase. The reaction is incubated at 37 °C for 10 minutes and quenched by addition of 1/10 volume 3M Na-acetate (pH 4.5). The mixture is extracted with equal volume phenol/chloroform/isoamylalcohol (25:24:1, pH 4.5) and once with chloroform/isoamylalcohol (24:1) followed by ethanol precipitation and lyophilized. The charged tRNA is resuspended in 2 mM Na-acetate pH 4.5 and stored at -80 °C. Example of a complete tRNA charging protocol is shown in figure 26.

15

20

Figure 27 shows the individual charged tRNAs. In vitro translation using PURE (Shimizu, 2001) requires the purification of coupled ribosomes. Coupled ribosomes are purified from *E. coli* strain MRE600 grown in LB-broth at to an OD450 of 0.5. Cells are snap-cooled on ice and harvested. The cell pellet is resuspended in 10 mM Tris-HCl pH 7.5, 10 mM Mg-acetate, 100 mM ammonium acetate and 6 mM mercaptoethanol. 10 mg/ml of lysozyme is added and the sample is snap-frozen and thawed on ice (3 times). Insoluble particles are pelleted by centrifugation and the supernatant is loaded onto 5-20 % sucrose gradients in a ribosome buffer: 20 mM Tris-HCl, pH7.5, 15 mM Mg-acetate, 100 mM ammonium acetate. Gradients are ultracentrifuged at 28.000 rpm for 14 hours and the ribosome profile in the gradient is monitored by UV-absorbance. Fractions containing 70S ribosomes are pooled

30

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and collected by ultracentrifugation. The purified coupled ribosomes are resuspended in ribosome buffer and stored at -80°C .

5 Additional proteins and enzymes required for PURE translation such as translation initiation factors 1-3, elongation factors G, Tu Ts, release Factors 1-3, ribosome recycling factor, methionineformyl transferase are purified as His-tagged versions from *E.coli* according to the procedure described by Shimizu et al., 2001. Other enzymes and reagents are available from commercial sources.

10 The constituents of a PURE translation mixture is shown below (Shimizu et al., 2001)

15	Coupled ribosomes (here, from <i>E. coli</i>)
	Initiation factors 1,2,3
	Elongation factors G, Tu, Ts
	Ribosome recycling factor (RRF)
	10 mM Mg-acetate
	5 mM K-phosphate pH 7.3
20	95 mM K-glutamate
	5 mM ammonium-chloride
	0.5 mM Calcium-chloride
	1 mM Spermidine
	8 mM putrescine
25	1 mM dithiothreitol (DTT)
	2 mM ATP
	2 mM GTP
	10 mM creatine phosphate
	0.5 μg 10 formyl-5,6,7,8-tetrahydrofolic acid
30	0.2 μg creatine kinase
	0.15 μg myokinase
	0.06 μg nucleoside-diphosphate kinase
	0.1 unit pyrophosphatase
	Enzymatically or chemically charged tRNAs
35	RNA template (DNA-puromycin tagged)

PURE translation mixture is incubated at 37°C for 1h.

- 5 Following translation, buffer, salts, nucleotides and other low molecular weight components are removed by gelfiltration. Furthermore, this step removes any translation products that are not coupled to their template sequence via the puromycin-linker.
- 10 Complexes consisting of translation products fused to their template is purified using poly(dT)-sepharose (AmershamPharmacia) according to established protocols. In brief, the above reaction mixture is incubated with 1 milligram of poly(dT)-sepharose for complex binding via the poly(dA)-tail of the template in binding buffer [TEN₂₀₀, 10 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.5] at 4 °C. Multiple washing steps
- 15 using binding buffer followed by elution using high salt buffer [TEN₂₀₀₀, 10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl] allows for the purification of translation products fused to their corresponding template. Excess salt is removed by gel-filtration.

- 20 The single stranded mRNA templates are converted to doublestranded RNA/DNA duplexes by the synthesis of cDNA. cDNA synthesis is conducted by annealing an oligodeoxynucleotide of the following composition; 5'-GG27T-3', to its complementary sequence of the 3' DNA linker portion on the mRNA template, incubated for 1 hour at 42 °C in a reaction mixture of the following composition: 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 0.5 mM spermidine,
- 25 200µM each of dATP, dGTP, dCTP and dTTP. Buffers and low molecular weight reagents are removed by gel-filtration.

- 30 The functional entities (see figure 27) comprised within the translation product each contain reactive groups capable of being reacted with a reactive group of an adjacent or neighbouring functional entity or capable of reacting with a EDC/NHS to form a coupling between neighbouring carboxylic acids and amines producing a β-peptide molecule. The reaction is carried out in a reaction mixture of the following composition; 20 mM Na-borate pH 9.0, 50 mM KCl, 100 mM EDC and 10 mM NHS and reacted > 2 hours at 40 °C. Buffer and unreacted EDC/NHS is removed by

gelfiltration. The reaction scheme for bond formation between reactive functional groups is shown in figure 28.

5 Following bond formation between carboxylic acid and amine reactive groups an activation step is employed to eliminate multiple linkages between the functional entities and the peptide (spacer) backbone leaving a single linker intact. Here, the nitrophenyl linkages are cleaved by UV-irradiation at > 300 nm for 15 min at 4 °C using a xenon-lamp and polystyrene plastic protection as filter. This final activation step produce a library of templated molecules each linked to their template/cDNA
10 sequence.

The library of templated molecules is employed for the selection of ligands having desired properties. Here, we are interested in selecting ligands capable of binding gamma-aminobutyric acid (GABA) A receptor, subtype α , a receptor family involved
15 in facilitating sedation, amnesia and seizure protection etc. (Mohler et al., 2002). The receptor protein is obtained from a recombinant source by cloning the gene for GABA receptor A, subtype α , in the bacterial expression system pET as a non-fusion variant (Novagen, Inc). The receptor protein is expressed and purified from *E. coli* strain BL21 using established protocols. The purified recombinant GABA
20 receptor protein is attached covalently to a solid support matrix by chemical coupling between CNBr-activated sepharose 4B (AmershamPharmacia) and exposed amino groups on the receptor protein.

The GABA receptor A, subtype α , attached to the sepharose 4B is used as target in
25 the selection of ligands from the library of templated molecules. To eliminate ligands that bind the matrix a counterselection step is employed. In brief, the pool of templated molecules are poured onto a 50 ml column containing 10 ml of settled CNBr-sepharose 4B in a binding buffer [25 mM Tris-HCl (pH 7.5), 150 mM K-glutamate, 5 mM $MgCl_2$, 5 mM DTT]. Following elution, 20 ml of binding buffer is
30 added and the eluent is collected. The eluent is loaded onto the settled sepharose 4 B matrix displaying the linked recombinant GABA receptor A, subtype α . Following successive washing steps using binding buffer, ligands are eluted using using 10 ml of elution buffer [25 mM Tris-HCl (pH 7.5), 2 M NaCl, 5 mM DTT]. Salt and low molecular weight reagents are removed by gelfiltration followed by concentration
35 using lyophilization. The ligands comprising templated molecules linked to their

templates are resuspended in low salt buffer [50 mM Na-acetate (pH 4.5)]. 50 units of chymotrypsin (Roche) are added to remove remaining peptide (spacer) backbone attached to the template sequence and the mixture is incubated for 15 minutes at 50 °C. The reaction mixture is extracted twice with an equal volume of phenol (pH 6.5) followed by extraction with an equal volume of chloroform and finally precipitated using ethanol.

The precipitated templates are amplified by PCR to generate doublestranded DNA templates for transcription. The PCR reaction is conducted using the oligo deoxynucleotide (T7) 5'-
CCGGGATCCTGTAATACGACTCACTATAGGCTGATCGATTTCAGTACGGAGG-3'
and (PR) 5'-CCCGGGATTCCGGACTA-3' in a reaction mixture of the following composition: 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 250 µM each of dGT, dATP, dCTP and dTTP, 2 mM MgCl₂, and isolated templates. Following PCR, buffers and unincorporated deoxynucleotides are removed by gelfiltration before synthesis of RNA templates as described above. The RNA templates are used for a second cycle of tagging and translation followed by cDNA synthesis, polymerisation of functional entities, activation of the encoded molecules, counterselection and selection of ligands and finally PCR amplification of templates encoding selected ligands. This scheme is iterated until a limited number of different template sequences are represented in the selected pool (usually less than 1000 different candidates). In most cases 8-12 cycles will suffice.

Finally, templates sequences isolated after 8-12 amplification/selection cycles are identified by cloning and sequencing enabling the identification of the chemical composition of selected ligands. These ligand candidates may be further characterised using pharmacokinetic, pharmacodynamic, toxicologic and in vivo behaviour etc.

30

Description of the Figures

The following symbols are used in the following figures to indicate general characteristics of the system: In figures 10-24, a long horizontal line symbolizes a

spacer backbone. For clarity, in some of the figures only the polymerization step, not the activation step, has been included. Rx denotes functional groups.

Figure 1A: Templated synthesis of a linear molecule - The principle.

5 Figure 1A illustrates an example of a protocol for synthesis of a linear templated molecule. The protocol can be divided into 9 steps with the starting material being either DNA or RNA of desired sequence composition. i) RNA template synthesis, ii) addition of 3'-end DNA-puromycin linker iii) Incorporation by translation iv) complementation of RNA template (cDNA), v) polymerization/bond formation, vi) 10 activation, vii) selection/screening, viii) amplification, and ix) characterization. Template-directed RNA synthesis may be mediated by enzymes such as T7, T3 or SP6 RNA polymerase to yield translational competent RNA templates or by chemical synthesis.

15 A DNA-puromycin tag is added to the 3'-end of each RNA template according to the procedure disclosed by Roberts et al. 1997 which may include ligation using ligase and a DNA oligonucleotide (splint) or by psoralen cross-linking as described by Kurz et al 2000 (see also Figure 2). The puromycin-linker is used in order to facilitate the coupling between the peptide product and the nucleic acid template. Additional protocols for the coupling templated molecules and their templates are described in 20 the "Summary of the invention" section.

In a preferred aspect of this invention the incorporation of building blocks into a spacer backbone according to the template sequence is accomplished by ribosome mediated translation. Upon completion the spacer backbone is attached covalently to its template via the puromycin entity. Each spacer unit or a subset of spacer units 25 incorporated into the spacer backbone specifies functional entities according to the template sequence and comprise one or more functional entity reactive groups and a cleavable or selectively cleavable linker. The template comprises primer binding sites at one or both ends (allowing the amplification of the template). The remaining portion of the template sequence may be random, partly random or fixed. Annealing 30 of a primer to a fixed sequence in the template 3'-end denoted the priming region (PR) allows template complementation by Reverse transcriptase forming cDNA. Reactions between reactive groups of the functional entities are initiated in a step forming covalent bonds between adjacent functional entities. These bonds are separate from those bonds synthesised by the peptidyl transferase activity of the 35 ribosome forming the spacer backbone.

Activation involves cleaving some or all but one of the cleavable linkers that connect functional entities to the spacer backbone.

Selection or screening involves enriching the population of template-templated molecule pairs for a desired property.

- 5 Amplification involves producing more of the template-templated molecule pairs, by amplification of the template or complementing template, and producing more of the template-templated molecule pairs, for further rounds of selection/screening. This step does not require characterization of individual sequences but can be performed on the population of molecules. Following multiple rounds of selection/screening,
10 selected template-molecule pairs are sequenced and/or characterised.

Cloning and sequencing involves the cloning of the isolated templates or complementing templates, followed by characterization. In some cases, it may be desirable to sequence the population of isolated templates or complementing templates, wherefore cloning of individual sequences are not required.

15

Figure 1B. Templated synthesis of a branched molecule.

The templated synthesis of a branched molecule can be produced using a principle similar to that described in the legend to figure 1A. Functional entities are incorporated into a spacer backbone according to the template sequence. The
20 scaffold is attached either covalently or non-covalently to the template that templated the formation of a spacer backbone comprising functional entities.

- Reactive groups of said functional entities are capable of reacting thereby forming a covalent bond with the scaffold resulting in a templated molecule comprised of functional entities as substituents on a central scaffold. The covalent attachment
25 between reaction between each functional entity and the scaffold may be directional (i.e. each subgroup of functional entities is only capable of reacting with one specific position on the scaffold) or a random process (all functional entities can react with any of the positions on the scaffold).

- 30 **Figure 1C: Example of a FE-units and non-FE-units displayed on a spacer backbone connected to its cognate template.**

Figure 2. A method used for retro-genetic tagging of peptides (PROFusion).

Steps A-C are schematic representations of steps involved in the production of RNA-protein fusions. **A**, illustrates a sample DNA construct for generation of a RNA portion of a fusion. **B**, illustrates the generation of an RNA/puromycin conjugate. **C**, illustrates the generation of an RNA-protein fusion.

5

Figure 3. Examples of non-standard amino acids and pseudo-amino acids capable of being incorporated into peptides.

Examples of amino acid-like entities known to be incorporated into peptides by in vivo or in vitro translation.

10

Figure 4: Building blocks:

A: Example of a first building block comprising a complementing element, a functional entity, a cleavable linker and spacer reactive groups.

B: Example of a second building block comprising a complementing element and spacer-reactive groups. The shown first building block comprises a tRNA charged with FE-AA unit capable of being incorporated into a spacer-backbone by ribosome mediated translation. Subsequent to the synthesis of the spacer-backbone the functional entity can participate in the formation of a templated molecule producing an α,β disubstituted β -peptide.

C: Examples of building blocks comprising FE-AA units. Shown is the 3'-terminal adenosine of a tRNA (complementing element) charged with FE-AA units via a covalent bond involving the adenosine 3' or 2' hydroxy-group. Below are listed examples of conditions used for bond formation between neighbouring FE-AA and for cleavage of cleavable or selectively cleavable linkers. 1) "fill-in" homobifunctional activated esters, cleavage by nucleophile; 2) Carboxyanhydride activated for step-by-step polymerisation by a primary amine, cleavage by nucleophile. 3) "fill-in" by homobifunctional activated esters, photocleavage; 4) Thiocarboxyanhydride activated for step-by-step polymerisation, cleavage by nucleophile; 5) Coumarin based coupling by photoactivation, cleavage by catalysis (H_2/Pd); 6) Double activated phosphoester unit. Coupling using dihydroxylated such as 1.3 dihydroxypyrimidine and enzymatic cleavage of linkers by chymotrypsin. 7) "fill-in" using activated bifunctional esters, photocleavage of linkers; 8) "fill-in" by activated esters, linker cleavage by elevated temperature; 9) Coupling by "fill-in" by ketone-hydrazide reaction and by modified Staudinger, linker cleavage by nucleophile; 10) "fill-in" coupling using double-activated esters, photocleavage of linkers. 11) Direct

- coupling using EDC/NHS; 12) "fill-in" coupling using double activated esters, linker cleavage by nucleophile; 13) "fill-in" by diamine and EDC/NHS activation of carboxy groups, linker cleavage by acid treatment; 14) "fill-in" by pericyclic coupling f. ex. By 1.4 benzoquinone, linker cleavage by nucleophile; 15) α,β disubstituted β -amino acid precursor, coupling by ring opening. Activated by a primary amine, linker cleavage by reduction of disulphide bridge (f.ex by DTT addition); 16) β -amino acid precursor capable of being translocated upon ester reaction with an adjacently positioned or a neighbouring amine of an adjacently positioned or neighbouring FE-AA, thus, coupling and linker cleavage occurs in the same reaction step; 17) Stepwise coupling of carboxyanhydride that are activated by a primary amino group. The "traceless" linker is photocleaved. 18) Scaffold molecule with reactive groups and no cleavable linker.

Figure 5: Charging of a complementing entity.

- 15 A: Enzymatic charging of a complementing entity by aminoacylation using amino acid tRNA synthetases for catalysis. Enzymatic charging may be carried out using wt, modified or mutant aminoacyl-tRNA synthetases. Alternatively other entities capable of charging a complementing entity such as selected or engineered RNA or DNA aptamers may conduct the charging of a complementing element.
- 20 B: Chemical charging of a complementing entity. Shown is the chemical charging of a tRNA using a 2-step protocol described by Mendel et al., 1995. A tRNA, lacking the essential 3' proximal CpA dinucleotide (cytidine-phospho-adenosine) is synthesised in vitro using T7 RNA polymerase and a DNA template comprising a promoter for T7 RNA polymerase transcription and the corresponding tRNA
- 25 sequence (minus 3'-end CpA). A pdCpA dinucleotide is synthesised using phosphoramidite chemistry and charged with an FE-AA entity carrying a convenient protection group(s) such as Boc, NVOC or, Foc.
- The charged complementing entity occurs by water elimination resulting in an ester linkage between the FE-AA entity and the 2' or 3' hydroxyl group of the terminal
- 30 adenosine residue. The charged pdCpA dinucleotide is ligated to the 3'-end of the pre-tRNA segment and subsequently deprotected using acid, photocleavage or similar technique depending on the protective group(s). These steps enable the chemical charging of a tRNA comprising a specific complementing element (codon) with a specific (cognate) FE-AA entity or any entity such as amino acids, non-natural
- 35 amino acids or pseudoamino acids capable of being incorporated into a spacer-

backbone by ribosome-mediated translation. The first charging step shown involving addition of FE-AA, amino acid, non-natural amino acid or pseudo-amino acid to the pdCpA can be conducted using oligonucleotides other than the pdCpA. The charging can be conducted using mono, tri, tetra, penta, hexa, hepta, octa, nona, deca etc oligonucleotides of appropriate sequence. Thus, the extent of the pre-tRNA to be ligated to the charged oligonucleotide should be designed accordingly.

Figure 6: Bond formation and Activation

Example of bond formation and activation by cleavage of cleavable linkers, wherein a disubstituted β -peptide comprising two monomer units linked to the spacer backbone is formed. After translation of a RNA template the functional entities are displayed on a spacer backbone (shown here at every second position). Bond formation is initiated by deprotection of an amine group, for example by a photoactivation step. Next, the primary amine attacks the carbonyl group of a neighbouring N-thiocarboxy anhydride (NTA) forming an amide bond upon releasing CSO. This reaction produces a primary amine for a second bond formation step involving the neighbouring NTA-unit resulting in the formation of a β -peptide comprising two monomer units and a non-cleavable linker. Subsequently, the β -dipeptide is activated by cleavage of the ester linkages connecting the β -peptide units and the spacer backbone resulting in a β -dipeptide connected to the spacer backbone via a single selectively cleavable linker. The template that encodes the β -dipeptide may be attached to the spacer backbone at any suitable position, but preferably at the C-terminus of the spacer backbone. For simplicity, the template has been omitted from the figure.

Figure 7: Display of FE-units by incorporation into an α -helix.

A: helical view down the axis of an α -helix. FE-units are displayed on the same face of the helix such as in position 2, 6, 9 etc or preferably as closest neighbour in position 2, 6, 10 etc. according to the helix structure.

B: FE-units incorporated at every fourth position in an α -helix. The template is attached to the spacer backbone at any convenient position (here, at the C-terminus of the spacer backbone). Following bond formation and activation of the linked FE-units the templated molecule is formed. The templated molecule is attached to the spacer backbone by one or more linkers and physically attached to its cognate template via the spacer backbone. Following selection of templated molecules with

desired properties, the appended template is amplified and transcribed by RNA polymerase. Purified RNA is tagged by DNA/puromycin in the RNA 3'-end, re-purified and translated.

- 5 To prevent interference in the selection procedure it may be advantageous to remove the main portion of the spacer backbone. One method for linker removal is to incorporate a lysine or arginine residue in the spacer backbone N-terminal of the spacer backbone unit connecting the templated molecule and the spacer backbone.

Figure 8: Display of functional entities by a coiled-coil structure.

- 10 Translation of a template will produce a spacer backbone (peptide) comprising FE-units. A subset of spacer backbone units incorporated into the template spacer backbone and which is not an FE-unit predispose the spacer backbone for coiled-coil structure formation. After translation, coiled-coil structure formation is initiated by interaction between two spacer backbones each comprising FE-units or between a
- 15 single spacer backbone comprising FE-units and a second spacer backbone (peptide) that does not contain FE-units. Such a second spacer-backbone may be prepared separately by solid-phase chemical synthesis, in vitro translation or by purification from cells of any source expressing any desirable spacer backbone capable of coiled-coil formation. Coiled-coil structure formation may also be induced
- 20 by intramolecular interaction between spacer segments separated by a turn structure. Following coiled-coil structure formation neighbouring FE-units are cross-linked. Subsequent cleavage of a subset of linkers produce a templated molecule linked to its template via at least one spacer unit. The template::templated molecule complexes with desired properties are selected and the templates are amplified
- 25 followed by transcription producing RNAs for DNA/puromycin tagging and subsequent translation.

Figure 9: Display of functional entities by a collagen triple-helix structure.

- 30 Translation of a template produce a spacer backbone comprising FE-units and spacer units enabling formation of a triple helix collagen-like structure. Subsequent to translation the triple helix structure is produced by interaction between three spacer backbones comprising FE-units or between one or two spacer backbones comprising FE-units and two or one spacer backbone not comprising FE-units, respectively. Such spacer backbones may be produced by solid-phase chemical
- 35 synthesis, in vitro translation or by purification from cells of any source expression

desirable spacer backbone molecules. Following triple helix formation FE-units are cross-linked. Cleavage of a subset of linkers produce a templated molecule, preferably connected to the spacer backbone by a single linker.

5 Template::templated molecule complexes with desired characteristics can be selected and their templates amplified enabling enrichment of templates encoding molecules with desired properties.

Figure 10. Cleavable linkers and protection groups.

10 Cleavable linkers and protection groups, agents that may be used for their cleavage and the products of cleavage.

Figure 11. Polymerization by reaction between neighbouring reactive groups.

15 For clarity, only the polymerization reaction (and not the activation) is shown in the figure. X represents the functional entity reactive groups. In this case the two reactive groups are identical.

Polymerization (reaction of X with X to form XX) either happens spontaneously when the monomer building block has been incorporated, or is induced by a change of conditions (e.g. pH), or by the addition of an inducing factor (chemical or UV exposure, for example)

Figure 11 example 1. Coumarin-based polymerization.

20 Light-induced reaction of the coumarin units, followed by activation (cleavage of the linker), results in a polymer backbone of aromatic and aliphatic ring structures. Examples of functional groups (phosphate, carboxylic acid and aniline) are shown.

Figure 12. Polymerization between neighboring non-identical reactive groups.

25 In this example, X may react with Y but not another X. Likewise, Y does not react with Y. Polymerization can either happen during the incorporation of building blocks (as shown in the figure), or after incorporation of several building blocks.

Figure 13. Cluster formation in the absence of directional polymerisation.

30 When the incorporated monomers are not fixed with regard to rotation about the linker bond that links the functional entities to the spacer backbone, cluster formation may result, as shown in the figure.

This represents a significant problem for longer polymers. The problem may be solved by (i) fixing the incorporated monomers in a preferred orientation which does not allow X and Y (reactive groups type II) to exchange positions in the array (e.g., by coupling the functional entity and the complementing element via a double bond or two bonds, e.g., coupling the functional entity to the C α -position and the amino group of the spacer backbone units, (ii) employing directional polymerisation ("zipping", see for example figure 17), or (iii) setting up conditions that ensure that the monomers react during or right after incorporation into the spacer backbone i.e., each monomer FE-unit reacts with the previously incorporated FE-unit before the next FE-unit is incorporated (see for example Figure 14, with example).

Figure 14. Zipping-polymerization and simultaneous activation.

Polymerization results in activation of the polymer. The geometry of the reaction between X and Y is in this example the same for all monomers participating in the polymerization

Figure 14, example 1. Simultaneous incorporation, polymerisation and activation - formation of peptides.

(A). Complementing entities specifying spacer backbone monomers to which amino acids thioesters have been appended, are incorporated into a spacer backbone. During or after incorporation of a spacer backbone monomer, the amine attacks the carbonyl of the (previously incorporated) neighbouring spacer backbone monomer. This results in formation of an amide bond, which extends the peptide one unit. When the next monomer is incorporated, this may attack the thioester carbonyl, resulting in cleavage of the dipeptide from the spacer backbone monomer, to form a tripeptide. The process continues further downstream the complementing template, until incorporation of monomers in the spacer backbone is arrested. Importantly, the geometry of the nucleophilic attack remains unchanged. As the local concentration of nucleophilic amines is much higher on the template than in solution, reactions in solution is not expected to significantly affect the formation of the correct templated molecule. Furthermore, the reactivity of the amine with the ester may be tuned in several ways. Parameters that will affect the reactivity include: (i) pH and temperature, (ii) length, point of attachment to the backbone spacer monomer, and characteristics (charge, rigidity, hydrophobicity, structure) of the linker that connects

the ester and the nucleotide, (iii) nature of ester (thio-, phospho-, or hydroxy-ester);
(iv) the nature of the substituent on the sulfur (see (B) below).

This general scheme involving incorporation, polymerisation and activation during or
right after incorporation of a FE-AA unit, can be applied to most nucleophilic
5 polymerisation reactions, including formation of various types of peptides, amides,
and amide-like polymers (e.g., mono-, di-, tri-, and tetra-substituted α -, β -, γ -, and Ω -
peptides, polyesters, polycarbonate, polycarbamate, polyurea), using similar
structures.

(B). Four different thioesters with different substituents and therefore different
10 reactivity towards nucleophiles.

**Figure 14, example 2. Simultaneous Incorporation, polymerization and
activation - formation of a polyamine.**

This figure shows a "rolling-circle polymerization reaction" where the chain
15 containing the nucleophilic center attacks the electrophile attached to the spacer
backbone using the spacer backbone as the leaving group.

Figure 15. "Fill-in" polymerization (symmetric XX monomers).

Fill-in polymerization by reaction between reactive groups (X in the figure) and
20 bridging molecules (Y-Y in figure).

For clarity, only the polymerization reaction (not the activation) is shown in the
figure. The thick line represents the spacer backbone. X represents the reactive
groups of the functional entity. In this case the two reactive groups are identical. (Y-
Y) is added to the mixture before, during or after incorporation of the FE-AA unit in
25 the spacer backbone. Likewise, significant reaction between X and Y may take
place during or after incorporation of the monomers.

Figure 15, example 1. Poly-imine formation by fill-in polymerization.

Dialdehyde is added in excess to incorporated diamines. As a result, a poly-imine is
30 formed. In the example, the polymer carries the following sequence of functional
groups: cyclopentadienyl, hydroxyl, and carboxylic acid.

Figure 15, example 2. Polyamide formation.

After incorporation into a spacer backbone of FE-AA unit containing diamines as
35 reactive groups, EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and

dicarboxylic acid is added in excess to the primary amines using standard coupling conditions. Alternatively, a di-(N-hydroxy-succinimide ester) may be added in excess, at a pH of 7-10. As a result, two amide-bonds are formed between two neighbouring FE-units. After this polymerisation, the appendices are separated from the spacer backbone (activation), leaving one linker intact, and the protected functional groups are deprotected to expose the functional groups. The final result is a template-spacer backbone-tagged polyamide.

An alternative route to polyamides would be to incorporate FE-AA units comprising di-carboxylic acids as reactive groups, and then add di-amines and EDC, to form amide bonds between individual FE-units. The backbone of the resulting polymer comprises or essentially consists of amide-bonded aromatic rings. The substituents of this example are a protected primary amine, a branched pentyl group, a tertiary amine and a pyrimidyl. The primary amine is protected in order to avoid its reaction with the dicarboxylic acid. Appropriate protecting groups would be for example Boc-, Fmoc, benzyloxycarbonyl (Z, cbz), trifluoracetyl, phthaloyl, or other amino protecting groups described e.g. in (T. W. Green and Peter G. M. Wuts (1991), Protective Groups in Organic Synthesis).

(B). The backbone comprises or essentially consists of aromatic rings, connected by amide bonds. The substituents are indanyl, diphenylphosphinyl, carboxamidoethyl and guanidylpropyl, the latter two representing the asparagine side chain, and the arginine side chain, respectively. The guanidyl function is protected, as it is more reactive than standard amines. An appropriate protecting group would be Mtr (4-methoxy-2,3,6-trimethylbenzenesulfonyl), Mts (mesitylene-2-sulfonyl) or Pbf (2,2,4,6,7-pentamethyldihydro-benzifuran-5-sulfonyl).

Figure 15, example 3. Polyurea formation.

Fe-AA units incorporated into a spacer backbone react with phosgen or a phosgen-equivalent such as CDI to form a polyurea. The linkers are cleaved and the protected hydroxyl is deprotected.

Appropriate leaving groups (Lv) are chloride, imidazole, nitrotriazole, or other good leaving groups commonly employed in organic synthesis

Figure 15, example 4. Chiral and achiral polyurea backbone formation.

In this example, the functional group Rx is used as a cleavable linker, that generates the desired functional group upon activation. In both (A) and (B), a polyurea is formed.

In (A), the functional group is attached to the backbone via a chiral carbon. The hydrogen on this carbon is drawn to emphasize this. Before polymerisation, there is free rotation about the bond connecting the chiral carbon and the functional group. When the reactive groups type II (the amines) react with the phosgen equivalent (e.g., a carbonyldiimidazole) to form the templated molecule, the FE-units may be inserted in either of two orientations (as indicated by the position of the hydrogen, left or right). As a result, each residue of the templated molecule has two possible chiral forms. Therefore, a given encoding molecule will encode a polymer templated molecule with a specific sequence of residues, but a templated molecule of 5 or 15 residues will have $2^5 = 32$ or $2^{15} = 32768$ stereoisomers, respectively. In certain cases it may be advantageous to incorporate such additional structural diversity in the library (for example when the templated molecule is relatively short). In other cases such additional diversity is not desirable, as the screening efficiency may become compromised, or it may become too difficult to deconvolute the structure of a templated molecule that has been isolated in a screening process, together with the other stereoisomers encoded by the same encoding molecule (for example when the templated molecule is long).

In (B), the chiral carbon of (A) has been replaced by a nitrogen. As a result, the resulting backbone of the templated molecule is achiral, and the encoding molecule encodes one specific structure.

Figure 15, example 5. Polyphosphodiester formation.

The incorporated nucleotide derivatives react with the activated phosphodiester to form a polyphosphodiester. Then the linkers are cleaved, resulting in a polyphosphodiester, attached through a linker to the encoding molecule. An example of an appropriate leaving groups (Lv) is imidazole.

Figure 15, example 6. Polyphosphodiester formation with one reactive group in each monomer building block.

Each incorporated nucleotide contains an activated phosphodiester. Upon addition of a dihydroxylated compound such as 1,3-dihydroxypyridine, a functionalised polyphosphodiester is formed. Finally, the functional groups Rx are liberated from

the complementing template by cleavage of the protection groups/cleavable linker that connected them to the oligonucleotide.

Figure 15, example 7. Pericyclic, "fill-in" polymerization.

- 5 After incorporation of FE-units in the spacer backbone 1,4-benzoquinone is added in excess, resulting in the formation of a polycyclic compound. Finally, the polymeric structure is activated by cleaving the linkers that connect the polymer to the spacer backbone, except for one (non-cleavable) linker which is left intact.

10 **Figure 16. "Fill-in" polymerization (asymmetric XS monomers).**

Fill-in polymerization by reaction between reactive groups ("X" and "S" in the figure) and bridging molecules (T-Y in figure).

- For clarity, only the polymerization reaction (not the activation) is shown. The thick line represents the spacer backbone. X and S represent the reactive groups of the functional entity. In this case the two reactive groups are non-identical. (T-Y) is added to the mixture before, during or after incorporation of the monomer building blocks. Likewise, significant reaction between X and Y, and between S and T may take place during or after incorporation of the backbone spacer monomer units.
- 15

20 **Figure 16, example 1. Fill-in polymerization by modified Staudinger ligation and ketone-hydrazide reaction.**

- The reactive groups X and S of the functional entities are azide and hydrazide. The added molecule that fills the gaps between the building blocks carry a ketone and a phosphine moiety. The reactions between a ketone and a hydrazide, and between a azide and a phosphine, are very chemoselective. Therefore, most functional groups Rx can be employed without the need for protection during the polymerization reactions. Examples for the molecular moieties R, R1, X and Y may be found in (Mahal et al. (1997), Science 276, pp. 1125-1128; Saxon et al. (2000), Organic Letters 2, pp. 2141-2143).
- 25

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Figure 17. "Zipping" polymerization.

- The initiator molecule (typically located at one of the ends of the nascent polymer) is activated, for example by deprotection or by a change in pH. The initiator then reacts with the reactive group X of the neighbouring unit. This activates the reactive group Y for attack on the neighbouring X. Polymerisation then travels to the other
- 35

end of the molecule in a "zipping" fashion, until all the desired monomers have been connected. The activation of the initiator (and reactive groups Y) may be both for attack by it on the neighbouring reactive group, or activation of it for attack by the neighbouring reactive group.

5

Figure 17, example 1. Radical polymerisation.

The initiator molecule, an iodide, is activated by the addition of a radical initiator, for example ammonium persulfate, AIBN (azobis-isobutyronitrile) or other radical chain reaction initiators. The radical attacks the neighboring monomer, to form a new radical and a bond between the first two monomers. Eventually the whole polymer is formed, and the polymer may be activated, which simultaneously creates the functional groups Rx.

10

Figure 17, example 2. Cationic polymerisation.

A cation is created by the exposure of the array to strong Lewis acid. The double bond of the neighbouring monomer reacts with this cation, whereby the positive charge migrates to the neighbouring monomer. Eventually the whole molecule is formed, and finally it is activated.

15

Figure 18. Zipping polymerization by ring opening.

The initiator reacts with the reactive group X in the ring structure, which opens the ring, whereby the reactive group Y in the same functional entity is activated for reaction with a reactive group X in a neighboring functional entity.

20

Figure 18, example 1. "Zipping" polymerization of N-thiocarboxyanhydrides, to form β -peptides.

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After incorporation of the building blocks, the initiator is deprotected. The primary amine then attacks the carbonyl of the neighbouring N-thiocarboxyanhydride (NTA) unit. As a result, CSO is released, and a primary amine is generated. This amine will now react with the next NTA unit in the array, and eventually all the NTA units will have reacted, to form a β -peptide. Finally, the templated molecule is activated. A number of changes to this set-up can be envisaged. For example, instead of thiocarboxyanhydrides, one might use carboxyanhydrides. The initiator might be protected with a base- or photolabile group. If a base-labile protection group is chosen, the stability of the carboxyanhydride must be considered. At higher pH it

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may be advantageous to use carboxyanhydrides rather than thiocarboxyanhydrides. Finally, the initiator might be unprotected and incorporated into the spacer backbone such as a lysine residue. In this case the concentration of the initiator in solution will be very low (typically nanomolar to micromolar), wherefore only an insignificant amount of initiator will react with the carboxyanhydrides. After or during incorporation of the building blocks the local concentration of initiator and carboxyanhydride will be much higher, leading to efficient polymerization. To avoid adverse side-reactions it may be beneficial if the spacer reactive groups of spacer backbone monomer units does not contain a primary amine (i. e. the amino-group of an amino acid can be substituted for an N-methyl amino acid, a hydroxyl- or thio-group, thus, preventing premature activation NTA or NCA units. Other types of peptides and peptide-like polymers (e.g., mono-, di-, tri-, and tetra-substituted α -, β -, γ -, and Ω -peptides, polyesters, polycarbonate, polycarbamate, polyurea) can be made, using similar cyclic structures. For example, α -peptides can be made by polymerization of 5-membered carboxyanhydride rings.

Figure 18, example 2. "Zipping" polymerization of 2,2-diphenylthiazinanone units to form β -peptides.

The deprotected nucleophile, a primary amine, attacks the carbonyl of the neighboring thioester, thereby forming an amide bond. The released thiol reorganizes, to form a thio-ketone. As a result a free primary amine is generated, which attacks the carbonyl of a neighbouring thioester, etc. Eventually an α -substituted β -peptide is formed, linked through its C-terminal end. The reactivity of the primary amine with the ester may be modified for example by the choice of ester (thioester or regular ester), pH during the polymerization reaction and the choice of substituents on the aromatic ring(s). The relative reactivity of the secondary amine contained in the cyclic moiety and the primary amine released upon ring-opening, may be adjusted by the bulk at the carbon between the secondary amine and the thioester. For example, replacing the two aromatic rings with one aromatic ring will decrease the bulk around the secondary amine, making it more nucleophilic, whereas the nucleophilicity of the primary amine that is formed upon ring-opening is not affected by the bulk at this position. Other peptides and amide-like polymers may be formed by this principle. For example, γ -peptides may be formed by polymerization of 7-membered thiazinanone rings.

Figure 18, example 3. Polyether formation by ring-opening polymerisation.

5 The initiator is deprotected by for example base or acid. The formed anion then attacks the epoxide of the neighboring monomer, to form an ether-bond. As a result, an anion is formed in the neighbouring unit. This attacks the next monomer in the array, and eventually the full-length polyether has been formed. Depending on the conditions the attack will be at the most or least hindered carbon of the epoxide (under acidic or basic conditions, respectively).

10 In the final step, the encoded polyether is activated. In this case, the polymer is fully released from the encoding molecule. The screening for relevant characteristics (e.g., effect in a cell-based assay or enzymatic activity) may be performed in microtiter wells or micelles, each compartment containing a specific template molecule and the templated polyether, in many copies. In this way, the template and templated molecule is physically associated (by the boundaries of the

15 compartment), and therefore the templates encoding polyethers with interesting characteristics may be collected from those compartments, pooled, amplified and "translated" into more copies of polyethers which may then be exposed to a new round of screening.

20

Figure 19. Zipping-polymerization and activation by rearrangement.

The initiator is activated for attack by Y. Reaction of initiator and Y results in release of the initiator from the complementing element. Upon reaction with the initiator, a rearrangement of the building block molecule takes place, resulting in activation of X

25 for reaction with Y. After a number of reactions and rearrangements, a polymer has been formed.

Figure 20. Zipping-polymerization and activation by ring opening.

Reaction of the initiator with X in the ring structure opens the ring, resulting in activation of Y. Y can now react with X in a neighbouring or adjacently positioned functional entity. As a result of ring-opening, the functional entities are released from the complementing elements.

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Figure 21. Directional polymer formation using fixed functional units.

- (A) The functional entity of a building block may be attached to the complementing element through two bonds. This may fix the functional entity in a given orientation relative to the spacer backbone. As a result, rotation around the linker that connects a functional entity and the spacer backbone (as depicted in figure 13) is not possible, and cluster formation therefore unlikely.
- (B) Incorporation of such conformationally restricted FE-units will position the amine (X in (A) above) in proximity to the ester (Y in (A) above). This ester may be activated, for example as an N-hydroxysuccinimide ester. After reaction of the amine and the ester, a polypeptide is formed. This polypeptide will be a directional polymer, with N-to-C-terminal directionality. In the present case, the polymerisation reaction will cleave the ester from the spacer backbone to which it is linked. Rotational fixation of the functional entity relative to the complementing element may be achieved in other ways. For example, the functional entity may be coupled to the spacer backbone through a double bond to C α of the spacer backbone monomer unit or it may be attached through one bond connecting C α and the functional entity and the amino terminus of a spacer backbone monomer unit.

Figure 22. Templated molecules.

- A non-exhaustive and non-limiting list of molecules that may be templated by the various principles described in the present invention. The list refers to any linear, branched or cyclic structure that contains one or more of the backbone structures listed, and/or contain several bonds of the same kind (e.g. amide bonds). Heteropolymers (hybrids of different polymer types) can also be templated by the present invention.

Figure 23. Reactive groups (polymer precursors).

A list of some of the precursors that may be used in the templated synthesis of various templated molecules.

Figure 24. Functional groups.

- A list of some of the functional groups, R_x (functionalities), that may be used with the templating schemes in the present invention. The functional groups may have to be protected during incorporation, polymerization, and/or activation, or may have to be introduced post activation.

Figure 25. Polymers and the functional entities required to make them.

The table provides examples of polymers that may be templated according to the principles described in the present invention. For each polymer, a suggested set of reactive groups (of the functional entity), a linking molecule or catalyst for the polymerization reaction where appropriate, are provided.

Figure 26: Example of a protocol for the chemical synthesis of charged tRNAs.**Figure 27: Examples of charged tRNAs (building blocks) for the synthesis of a library of templated β -peptide molecules.**

(A) An overall structure of a charged tRNA composed of a RNA segment containing an unspecified anticodon sequence (NNN) charged with an unspecified functional entity FE_x attached to the RNA segment via the amino acid (spacer) unit. Each specific anticodon sequence corresponds to a specific functional entity. The remaining part of a charged tRNA may be identical for all building blocks. Examples of specific anticodon sequences and their corresponding functional entities are shown in (B).

Figure 28: Bond formation between amines of functional entities arrayed on a peptide (spacer) backbone structure. Bond formation is accomplished by EDC/NHS facilitated coupling between $-NH_2$ and $-COOH$ groups**Figure 29. Examples of pairs of reactive groups (X) and (Y), and the resulting bond (XY).**

Non-limiting examples of reactive groups, in particular functional entity reactive groups are shown, along with the bonds formed upon their reaction. After reaction, activation (cleavage) may be required (see Figure 29).

Claims

1. A method for synthesising a templated molecule comprising a plurality of functional groups, said method comprising the steps of
- 5
- i) providing at least one template comprising a sequence of n coding elements selected from the group consisting of first coding elements and second coding elements,
- 10
- wherein each coding element comprises at least one recognition group capable of recognising a predetermined complementing element, and
- wherein n is an integer of at least 3,
- 15
- with the proviso that the template comprises at least 3 first coding elements,
- ii) providing a plurality of building blocks selected from the group consisting of first building blocks and second building blocks, with the proviso that at
- 20
- least 3 first building blocks are provided,
- wherein each first building block comprises
- a) at least one complementing entity comprising a first complementing
- 25
- element comprising at least one recognition group capable of recognising a predetermined first coding element,
- b) at least one functional entity comprising at least one functional group and at least one functional entity reactive group, and
- 30
- c) at least one spacer comprising at least one spacer reactive group, wherein the spacer is separating the at least one functional entity from the at least one complementing entity, and
- 35
- wherein each second building block comprises

- 5 a) at least one complementing entity comprising a second complementing element comprising at least one recognition group capable of recognising a predetermined second coding element,
- b) and at least one spacer comprising at least one spacer reactive group,
- 10 iii) complementing coding elements by contacting each coding element with a building block complementing element capable of recognising said coding element, wherein at least 2 coding elements are interacting with complementing elements simultaneously,
- with the proviso that a total of at least 3 first coding elements are complemented; and
- 15 iv) forming a spacer backbone by linking neighbouring spacers in a ribosome catalysed reaction by means of reacting spacer reactive groups, and
- 20 v) obtaining a templated molecule comprising at least 3 covalently linked, functional groups by linking, by means of reacting functional entity reactive groups, a functional group of one functional entity to a functional group of another adjacently positioned, functional entity and linking said
- 25 other functional entity to yet another adjacently positioned functional entity.

2. The method according to claim 1, wherein step iii) to iv) comprises the steps of

- 30 a) complementing 2 neighbouring coding elements simultaneously by contacting each coding element with a building block complementing element capable of recognising said coding element,
- b) forming a spacer backbone by linking, by means of a reaction involving
- 35 spacer reactive groups, the 2 building block spacers,

- c) complementing at least one further predetermined coding element by contacting said coding element with a building block complementing element capable of recognising said coding element, and
- 5 d) elongating the spacer backbone by linking to the spacer backbone, by means of a reaction involving spacer reactive groups, the neighbouring building block spacer.
- 10 3. The method according to claim 2, wherein the steps of the method are performed in the order mentioned.
- 15 4. Method of claim 2, wherein steps c) and d) are repeated at least twice, such as repeated at least three times, for example at least 4 times, such as at least 5 times, for example at least 10 times, for example at least 15 times, such as at least 20 times, for example at least 30 times, such as at least 40, for example at least 50, such as at least 75 times, for example at least 100 times, such as at least 150 times, for example at least 200 times.
- 20 5. Method of claim 2, wherein steps c) and d) are repeated between 2 and preferably 10,000 times, for example between 5 and preferably 1000 times, such as between 10 and preferably 500 times.
- 25 6. The method according to any of claims 1 to 5, which furthermore comprises the step of
- iva) breaking the covalent bond between the spacer backbone and at least one complementing element.
- 30 7. The method according to claim 6, wherein the step iva) is performed once after every performance of step iv) of claim 1 or once after every performance of step b) or d) of claim 2.
- 35 8. The method according to any of claims 1 to 7, which furthermore comprises the step of

- v) breaking the covalent bond between the spacer backbone and at least one functional entity.
9. The method according to claim 8, wherein the covalent bond is selected from the group consisting of cleavable linkers and selectively cleavable linkers.
10. The method according to claim 8, wherein all covalent bonds between the spacer backbone and the functional entities are broken except for one.
11. The method according to any of claims 1 to 10, wherein the template comprises a ratio of first coding elements to second coding elements of 50:1, such as 40:1, for example 30:1, such as 25:1, for example 20:1, such as 15:1, for example 10:1, such as 8:1, for example 6:1, such as 5:1, for example 4:1, such as 3:1, for example 2:1, such as 1:1, for example 1:2, such as 1:3, for example 1:4, such as 1:5, for example 1:6, for example 1:7, such as 1:8, for example 1:10, such as 1:15, for example 1:20, such as 1:25, for example 1:30, such as 1:40, for example 1:50.
12. The method according to any of claims 1 to 11, wherein the template comprises at least 1, for example at least 2, such as at least 3, for example at least 4, such as at least 5, for example at least 10, for example at least 15, such as at least 20, for example at least 30, such as at least 40, for example at least 50, such as at least 75, for example at least 100, such as at least 150, for example at least 200 elements first coding elements.
13. The method according to any of claims 1 to 12, wherein the template comprises at least 1 for example at least 2, such as at least 3, for example at least 4, such as at least 5, for example at least 10, for example at least 15, such as at least 20, for example at least 30, such as at least 40, for example at least 50, such as at least 75, for example at least 100, such as at least 150, for example at least 200 second coding elements.
14. The method according to any of claims 1 to 13, wherein the ribosome is a wild type ribosome.

15. The method according to any of claims 1 to 14, wherein the spacer backbone only comprises spacer residues that are directly attached to a functional entity.
- 5 16. The method according to any of claims 1 to 15, wherein the spacer backbone comprises spacer residues that are directly attached to a functional entity, wherein every two spacer residues that are directly attached to a functional entity are separated by a minimum of 0 spacer residues that are not directly attached to a functional entity, for example at least 1, such as at least 2 first, for
10 example around 2, such as around 3, for example around 4, such as around 5, for example around 6, for example around 7, such as around 8 to 10, for example around 10 to 15, such as around 15 to 20, for example around 20 to 30 spacer residues, that are not directly attached to a functional entity.
- 15 17. The method according to any of claims 1 to 16, wherein the spacer backbone has the form of an α -helix.
18. The method according to any of claims 1 to 17, wherein the spacer backbone has the form of a coiled coil.
- 20 19. The method according to any of claims 1 to 18, wherein the spacer backbone has a form selected from the group consisting of β -sheets, beta-turn, beta-helix, helix-turn helix, part of a collagen structure, or part of a zinc finger structure.
- 25 20. The method according to any of claims 1 to 19, wherein the spacer backbone is denatured and bound to a solid surface that determines the shape of the spacer backbone.
- 30 21. The method according to claim 17, wherein the spacer backbone comprises one functional entity per helical turn of the spacer backbone.
22. The method according to claim 17, wherein the spacer backbone comprises a functional entity for every 4 spacer residues.

23. The method according to any of claims 1 to 22, wherein n is an integer of more than 1 and less than 1000, for example between 5 and 500, such as between 10 and 100.
- 5 24. The method of any of claims 1 to 23, wherein the spacer backbone is a linear sequence of spacers.
25. The method according to any of claims 1 to 24, wherein the complementing entity is a tRNA like structure.
- 10 26. The method according to any of claims 1 to 25, wherein the complementing entity is a tRNA.
27. The method according to any of claims 1 to 26, wherein the complementing entity is a pseudoknot.
- 15 28. The method of any of claims 1 to 27, wherein the complementing elements are selected from the group consisting of nucleotides, nucleotide derivatives, nucleotide analogues, and any combination thereof.
- 20 29. The method according to any of claims 1 to 28, wherein each complementing element consists of 1 nucleotide, such as 2, for example 3, such as 4, for example 5, such as 5 to 10, for example 10 to 15, such as 15 to 20, for example more than 20.
- 25 30. The method of claims 28 or 29, wherein the nucleotides are ribonucleic acids comprising a base selected from adenine (A), uracil (U), guanine (G), and cytosine (C) and derivatives and analogues thereof.
- 30 31. Method according to any of claims 1 to 30, wherein the complementing element is an anticodon.
32. Method according to any of claims 1 to 30, wherein the template is nucleic acid.

33. Method according to any of claims 1 to 32, wherein the template is a nucleic acid, which can be template of a ribosome mediated translation.
34. Method according to claim 33, wherein the template comprises or consists of
5 RNA or a derivative or analogue thereof.
35. The method according to any of claims 32 to 34, wherein the template comprises RNA residues that are modified on the 2' position of the ribose moiety.
10
36. The method according to any of claims 1 to 35, wherein the template is capped RNA.
37. The method according to any of claim 1, wherein the template is mRNA.
15
38. The method according to any of claims 1 to 37, wherein the template is tethered to puromycin.
39. The method of any of claims 1 to 38, wherein the coding elements are selected
20 from the group consisting of nucleotides, nucleotide derivatives, nucleotide analogs, and any combination thereof.
40. The method according to any of claims 1 to 39, wherein each coding element consists of 1 nucleotide, such as 2, for example 3, such as 4, for example 5,
25 such as 5 to 10, for example 10 to 15, such as 15 to 20, for example more than 20.
41. The method of claim 40, wherein the nucleotides are ribonucleic acids comprising a base selected from adenine (A), uracil (U), guanine (G), and
30 cytosine (C) and derivates and analogues thereof.
42. Method according to any of claims 1 to 41, wherein the coding element is a codon.

43. The method according to any of claims 1 to 42, wherein the spacer is selected from the group consisting of amino acids.
44. The method according to any of claims 1 to 43, wherein the spacer is selected
5 from the group consisting of α -amino acids.
45. Method according to claim 43, wherein the amino acid is a standard amino acid residue or a derivative thereof.
- 10 46. The method according to any of claims 1 to 45, wherein the spacer consists of a naturally occurring amino acid residues including the entire side-chain and wherein the spacer does not form part of the functional entity.
- 15 47. Method according to claim 43, wherein the amino acid is a non-standard amino acid.
48. Method according to claim 43, wherein the amino acid is a modified standard amino acid.
- 20 49. The method according to any of claims 1 to 48, wherein each spacer comprises at least 1, such as 2, for example 3, such as more than 3 spacer reactive groups.
- 25 50. Method according to any of claims 1 to 49, wherein the spacer reactive groups are selected from the group consisting of acyls and amines.
- 30 51. The method according to any of claims 1 to 50, wherein each spacer comprises one spacer reactive group, which is an acyl and another spacer reactive group which is an amine.
52. Method according to any of claims 1 to 3, wherein linking according to step iv) consists of the formation of an amide-bond.
- 35 53. The method according to any of claims 1 to 3, wherein the adjacently positioned functional entities are positioned sequentially on the spacer backbone.

54. The method of any of claims 1 to 3, wherein the functional entities are selected from the group consisting of α -amino acids, β -amino acids, γ -amino acids, ω -amino acids.
55. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of amino acids selected from the group consisting of α -amino acids, β -amino acids, γ -amino acids, ω -amino acids.
56. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of α -amino acids.
57. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of monosubstituted α -amino acids.
58. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of disubstituted α -amino acids.
59. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of monosubstituted β -amino acids.
60. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of disubstituted β -amino acids.
61. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of trisubstituted β -amino acids.
62. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of tetrasubstituted β -amino acids.
63. The method of any of claims 59 to 62, wherein the backbone structure of said β -amino acids comprises or essentially consists of a cyclohexane-backbone and/or a cyclopentane-backbone.

64. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of γ -amino acids.
65. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of ω -amino acids.
66. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of vinylogous amino acids.
67. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of N-substituted glycines.
68. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of functional groups and/or functional entities selected from the group of α -peptides, β -peptides, γ -peptides, ω -peptides, mono-, di- and tri-substituted α -peptides, β -peptides, γ -peptides, ω -peptides, peptides wherein the amino acid residues are in the L-form or in the D-form, vinylogous polypeptides, glycopoly-peptides, polyamides, vinylogous sulfonamide peptide, polysulfonamide, conjugated peptides comprising e.g. prosthetic groups, polyesters, polysaccharides, polycarbamates, polycarbonates, polyureas, polypeptidylphosphonates, polyurethanes, azatides, oligo N-substituted glycines, polyethers, ethoxyformacetal oligomers, poly-thioethers, polyethylene glycols (PEG), polyethylenes, polydisulfides, polyarylene sulfides, polynucleotides, PNAs, LNAs, morpholinos, oligo pyrrolinone, polyoximes, polyimines, polyethyleneimines, polyimides, polyacetals, polyacetates, polystyrenes, polyvinyl, lipids, phospholipids, glycolipids, polycyclic compounds comprising e.g. aliphatic or aromatic cycles, including polyheterocyclic compounds, proteoglycans, and polysiloxanes, including any combination thereof.
69. The method of any of claims 1 to 3, wherein neighbouring residues of the templated molecule is linked by a chemical bond selected from the group of chemical bonds consisting of peptide bonds, sulfonamide bonds, ester bonds, saccharide bonds, carbamate bonds, carbonate bonds, urea bonds, phosphonate bonds, urethane bonds, azatide bonds, peptoid bonds, ether bonds, ethoxy bonds, thioether bonds, single carbon bonds, double carbon

bonds, triple carbon bonds, disulfide bonds, sulfide bonds, phosphodiester bonds, oxime bonds, imine bonds, imide bonds, including any combination thereof.

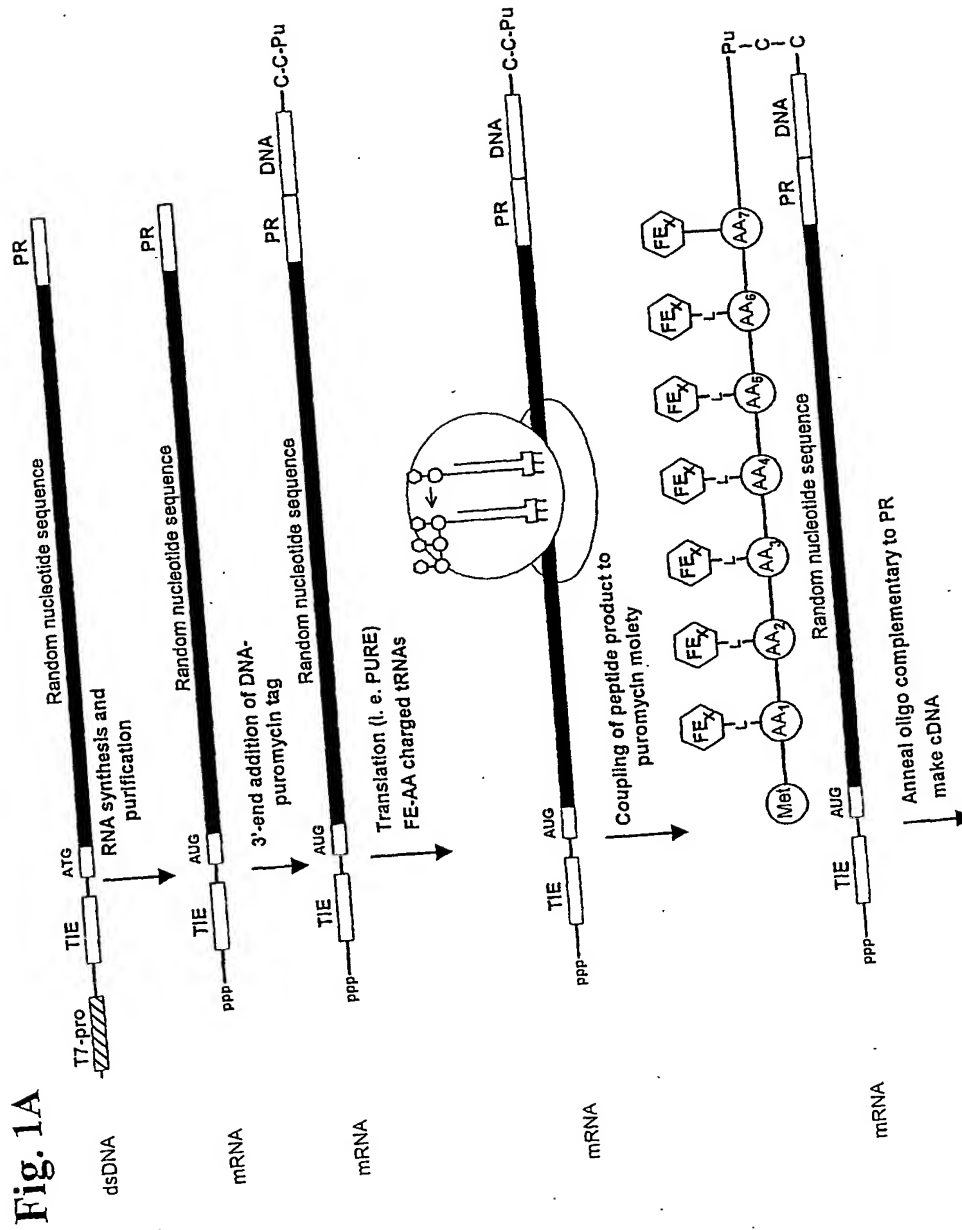
- 5 70. The method of any of claims 1 to 3, wherein the backbone structure of said templated molecule comprises or essentially consists of a molecular group selected from -NHN(R)CO- ; -NHB(R)CO- ; -NHC(RR')CO- ; -NHC(=CHR)CO- ; -NHC₆H₄CO- ; -NHCH₂CHRCO- ; -NHCHRCH₂CO- ; -COCH₂- ; -COS- ; -CONR- ; -COO- ; -CSNH- ; -CH₂NH- ; -CH₂CH₂- ; -CH₂S- ; -CH₂SO- ;
10 -CH₂SO₂- ; -CH(CH₃)S- ; -CH=CH- ; -NHCO- ; -NHCONH- ; -CONHO- ; -C(=CH₂)CH₂- ; -PO₂NH- ; -PO₂CH₂- ; -PO₂CH₂N⁺- ; -SO₂NH- ; and lactams.
- 15 71. The method of any of claims 1 to 3, wherein the templated molecule comprises or essentially consists of at least 2 different functional groups, such as at least 3 different functional groups, for example at least 4 different functional groups, such as at least 5 different functional groups, for example at least 6 different functional groups, such as at least 7 different functional groups, for example at least 8 different functional groups, such as at least 9 different functional groups, for example at least 10 different functional groups, such as more than 10
20 different functional groups.
72. The method of any of claims 1 to 3, wherein the functional groups are identical.
- 25 73. The method according to any of claims 1 to 3, wherein each functional entity comprises more than one, such as 2, for example 3, such as 4, for example 5, such as more than 5 functional entity reactive groups.
- 30 74. The method according to any of claims 1 to 3, wherein the functional entity reactive groups are selected from the group consisting of N-carboxyanhydride (NCA), N-thiocarboxyanhydride (NTA), amine, carboxylic acid, ketone, aldehyde, hydroxyl, thiol, ester, thioester, any conjugated system of double bonds, hydrazine, N-hydroxysuccinimide ester, and epoxide.
- 35 75. The method according to claim 74, wherein the functional entity reactive group is an electrophile.

76. The method according to claim 75, wherein the functional entity reactive group is a nucleophile.
- 5 77. The method according to claim 76, wherein the functional entity reactive group is a radical.
- 10 78. A template/templated molecule complex comprising a template and a templated molecule, wherein the template encodes the synthesis of the templated molecule and wherein said templated molecule comprises at least 3 covalently linked functional groups.
- 15 79. A template/templated molecule complex comprising a template and a templated molecule, wherein the template templates the synthesis of the templated molecule and wherein said templated molecule comprises at least 3 covalently linked functional groups, with the proviso, that the templated molecule is not a standard polypeptide.
- 20 80. The complex according to any of claims 78 and 79, wherein the complex furthermore comprises a spacer backbone.
- 25 81. The complex according to claim 80, wherein the spacer backbone is linked to the templated molecule by 1, such as 2, for example 3, such as more than 3 covalent bonds.
82. The complex according to any of claims 78 and 79, wherein the template is linked to the templated molecule via a puromycin linker.
- 30 83. A plurality of templated molecules, wherein the plurality comprises at least 1000 different templated molecules and wherein said templated molecule comprises a sequence of at least 3 functional groups, each encoded by a coding element of a template, with the proviso, that the templated molecule is not a standard polypeptide.

84. A plurality of template/templated molecule complexes comprising at least 1000 different template/templated molecule complexes, wherein each template/templated molecule complex is a complex according to any of claims 78 and 79.

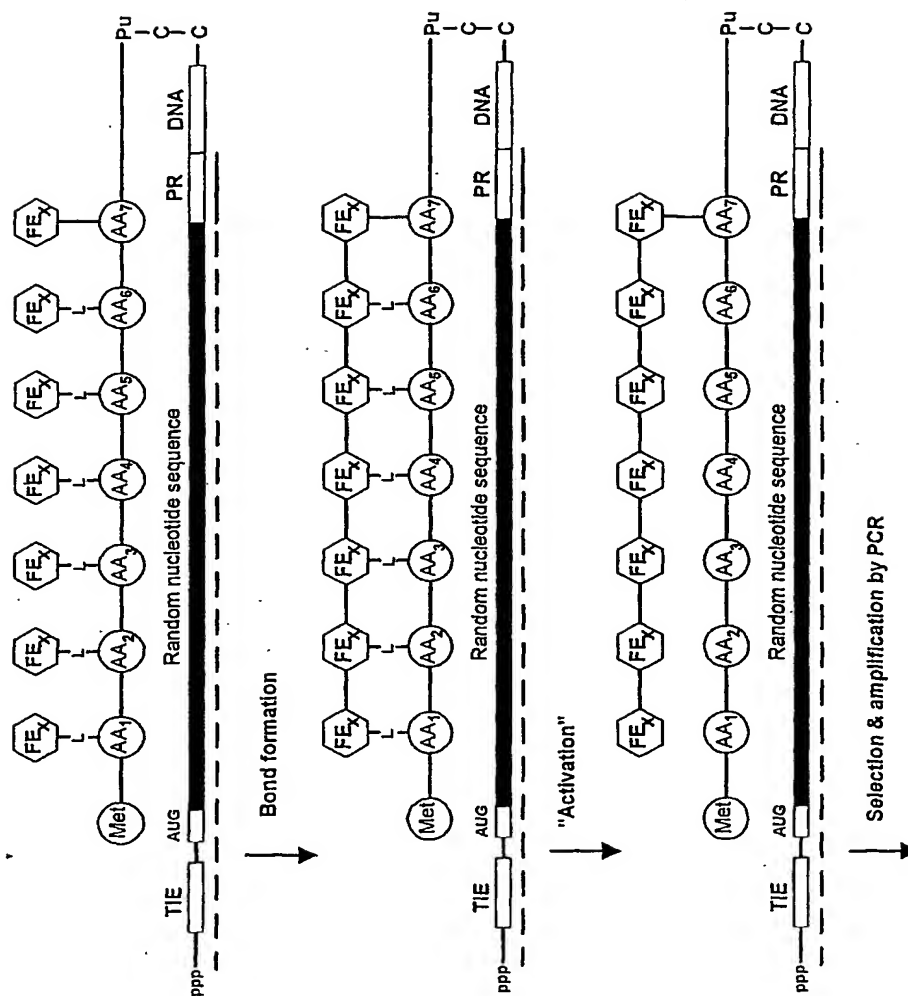
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Templated polymers - the principle



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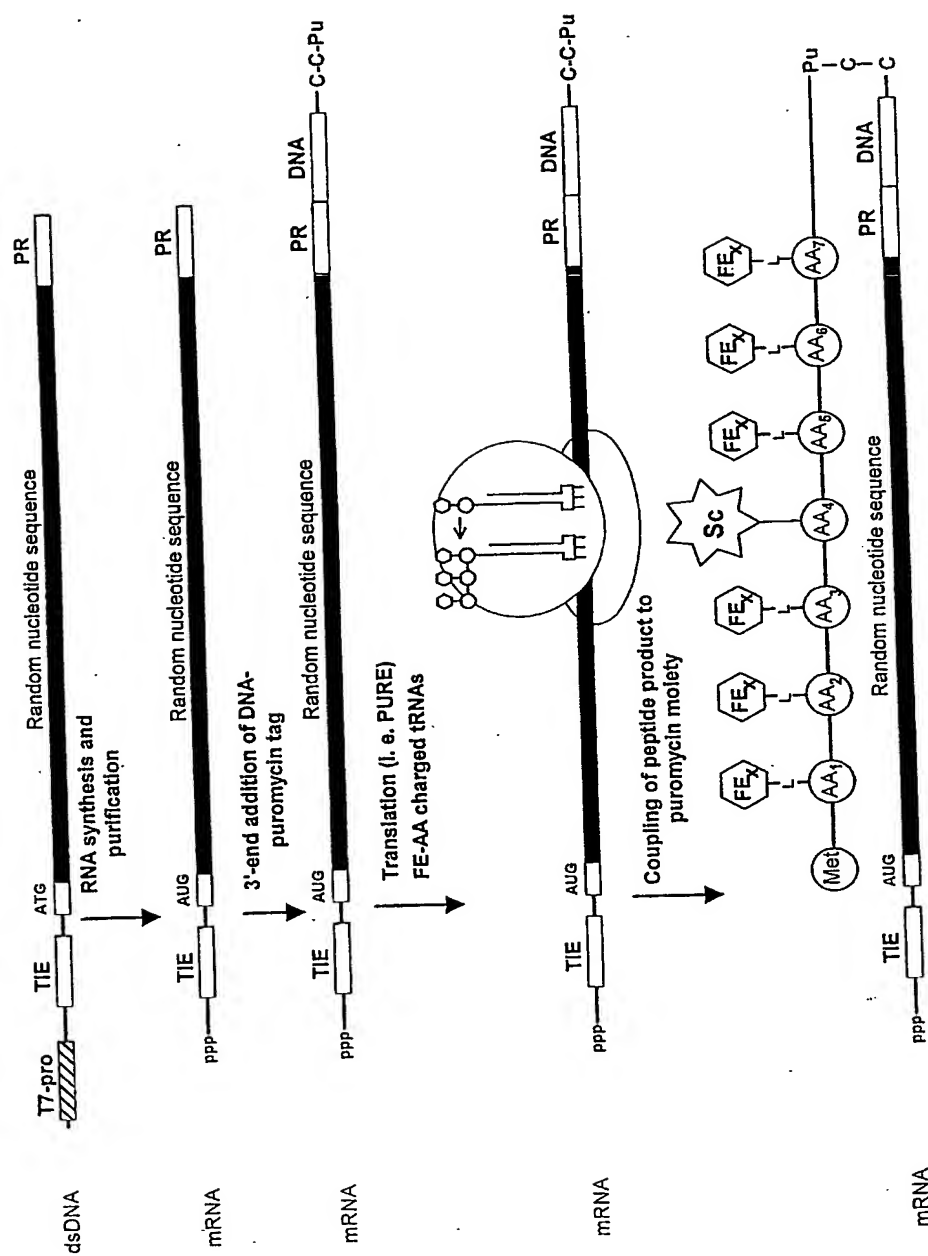
Fig. 1A, continued



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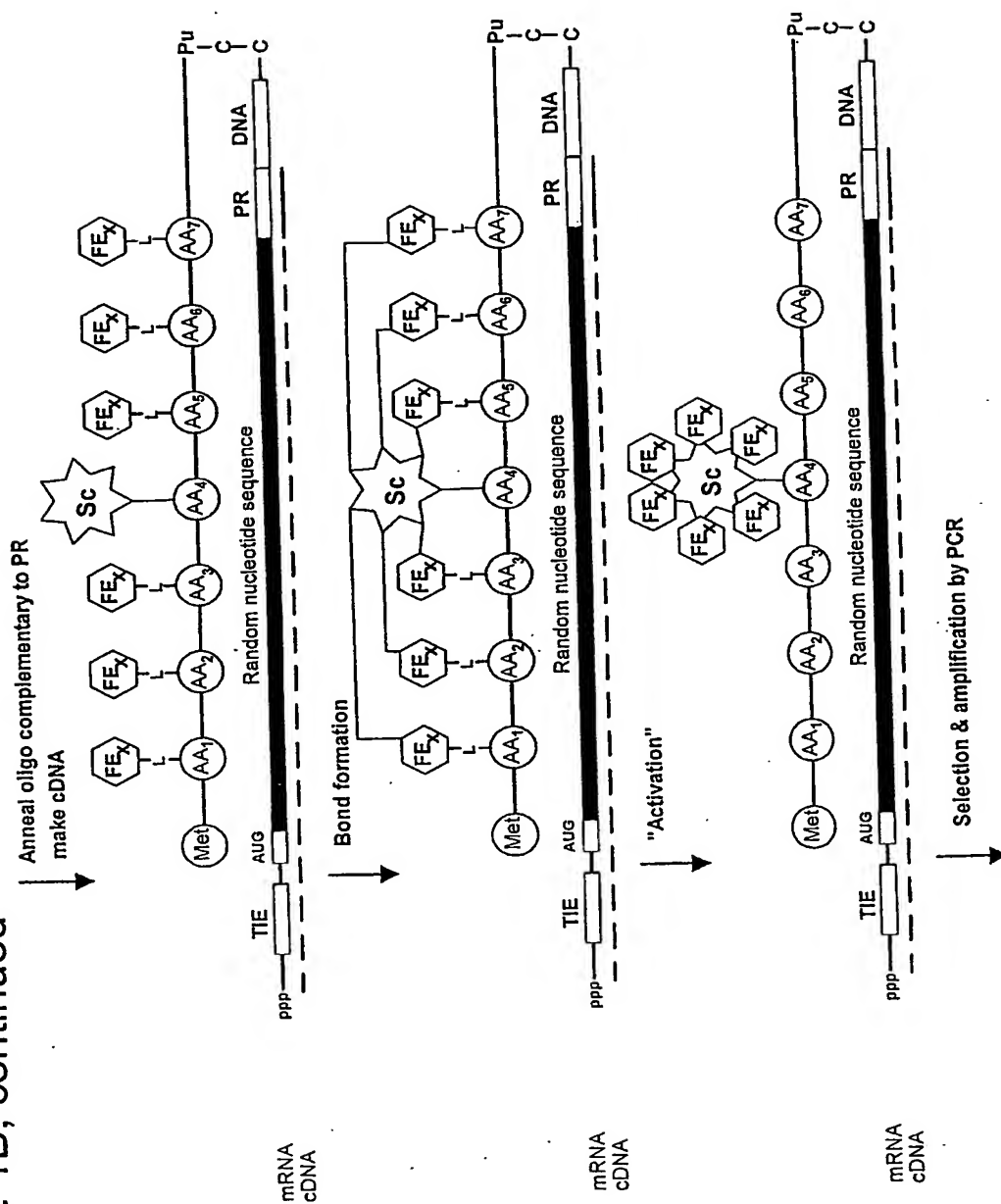
Templated branched molecules - the principle

Fig.1B



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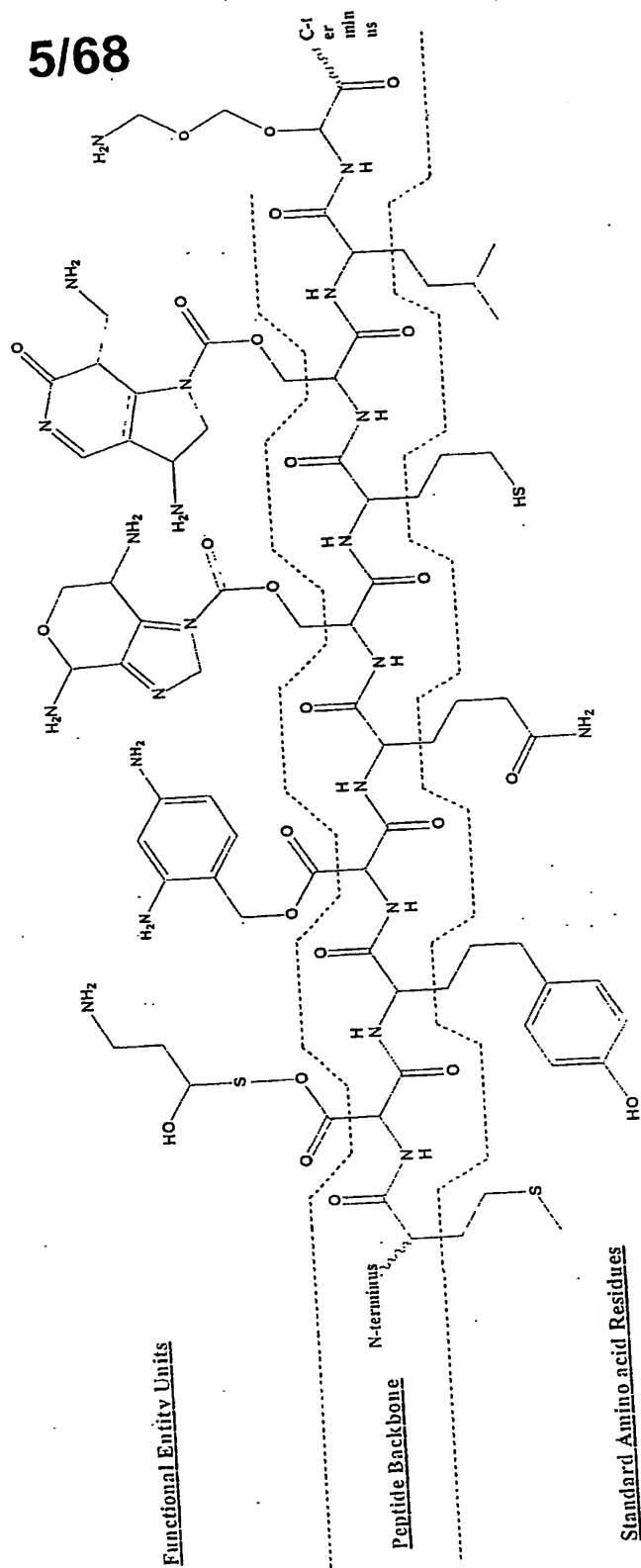
Fig. 1B, continued



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Fig. 1C

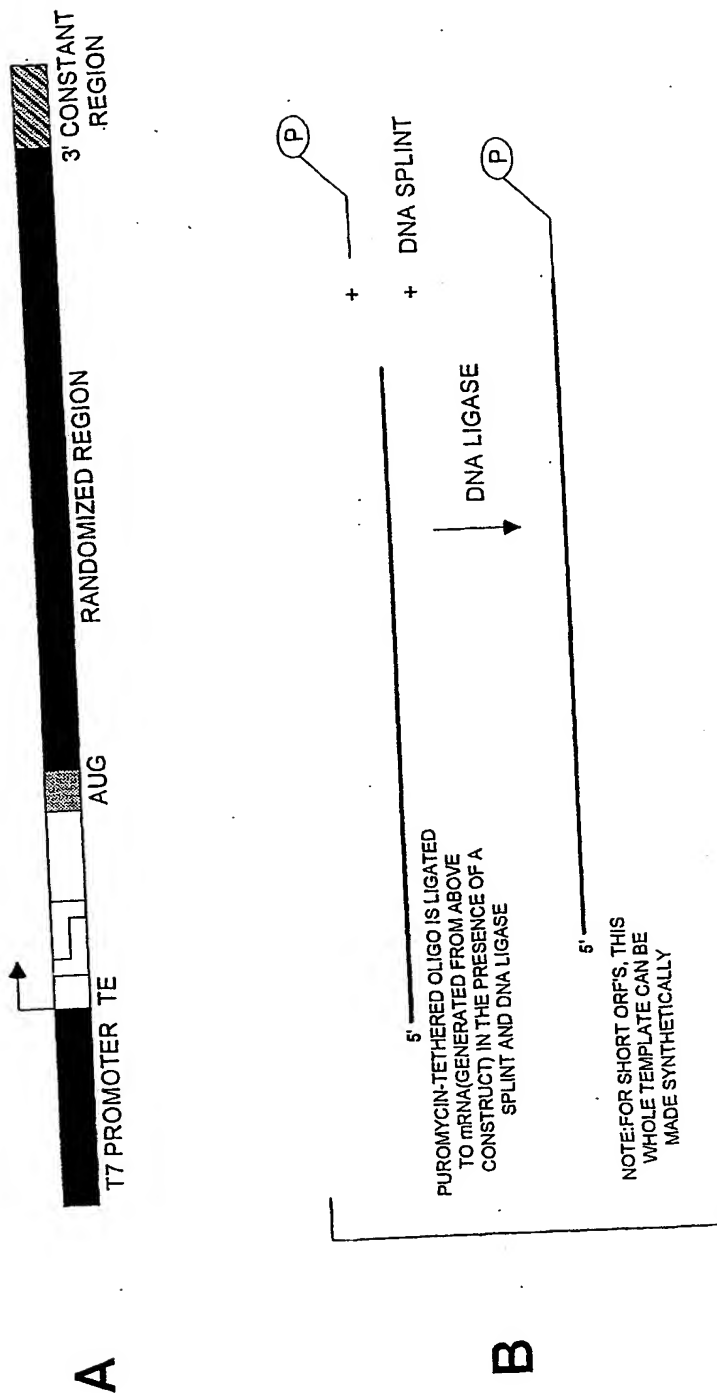
Display of Functional Entities on a Peptide Backbone



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PROFusion

Fig. 2



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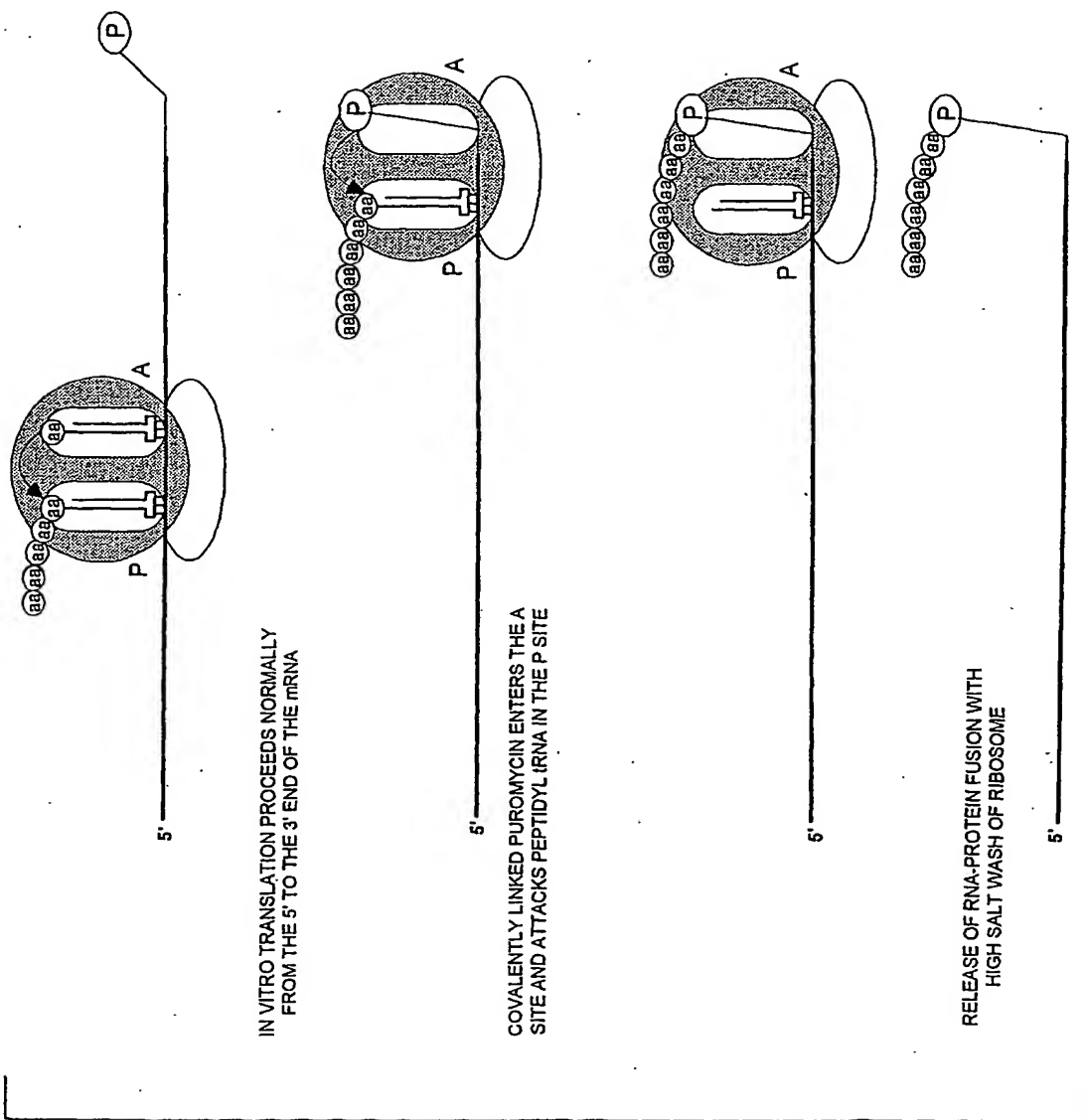
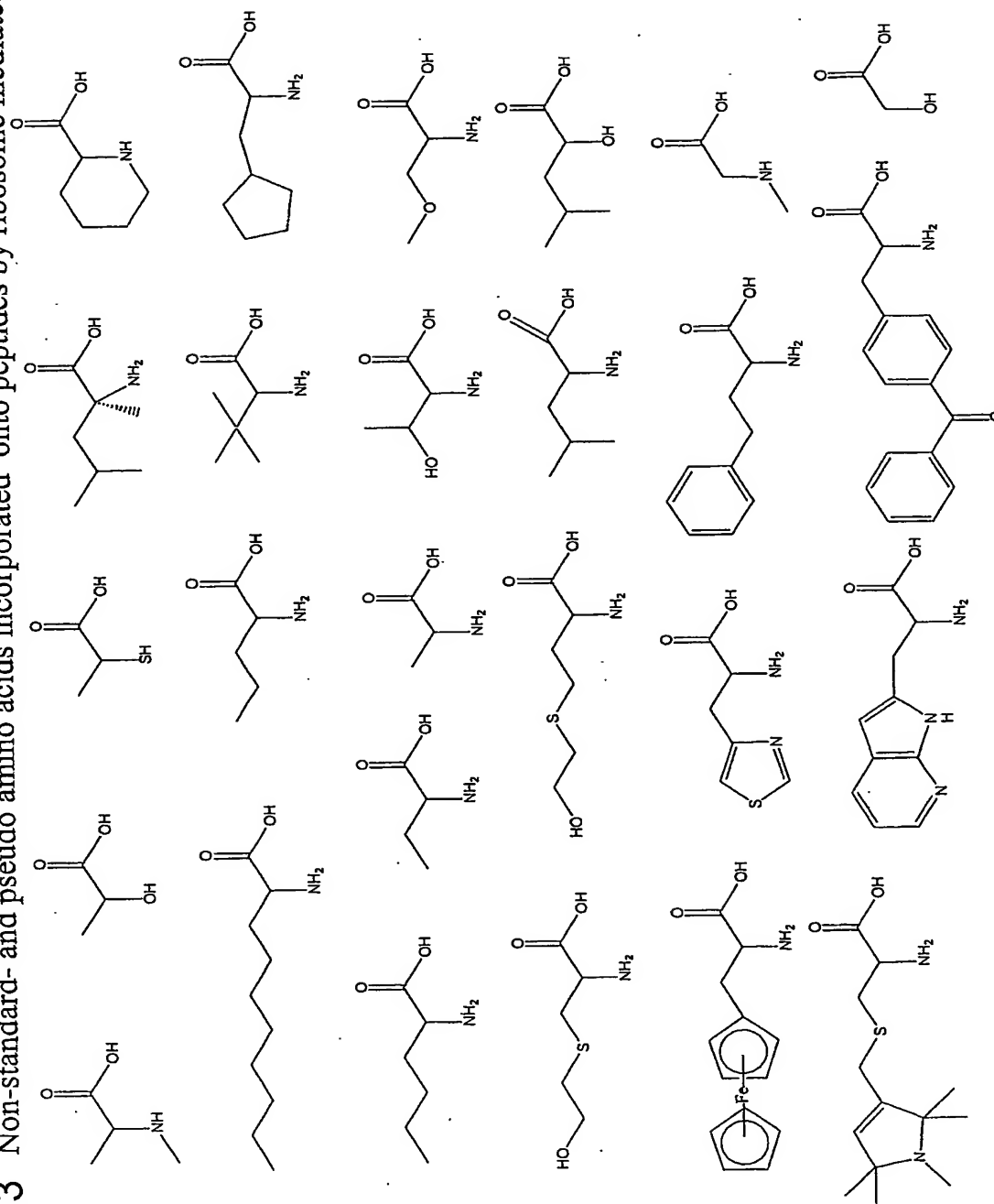


Fig. 2,
continued

C

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Fig. 3 Non-standard- and pseudo amino acids incorporated onto peptides by ribosome mediated translation.

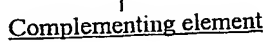


3, continued

Fig. 3, continued

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Example of a first building block



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Fig. 4B

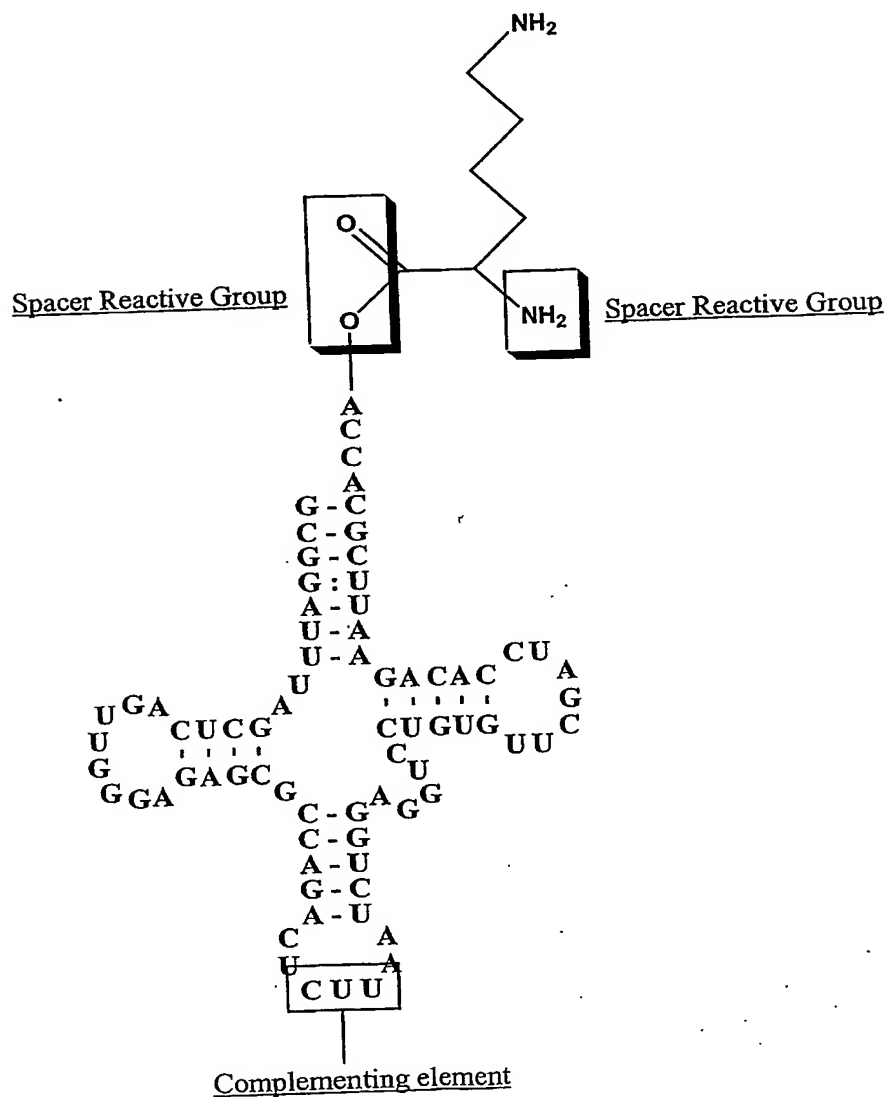
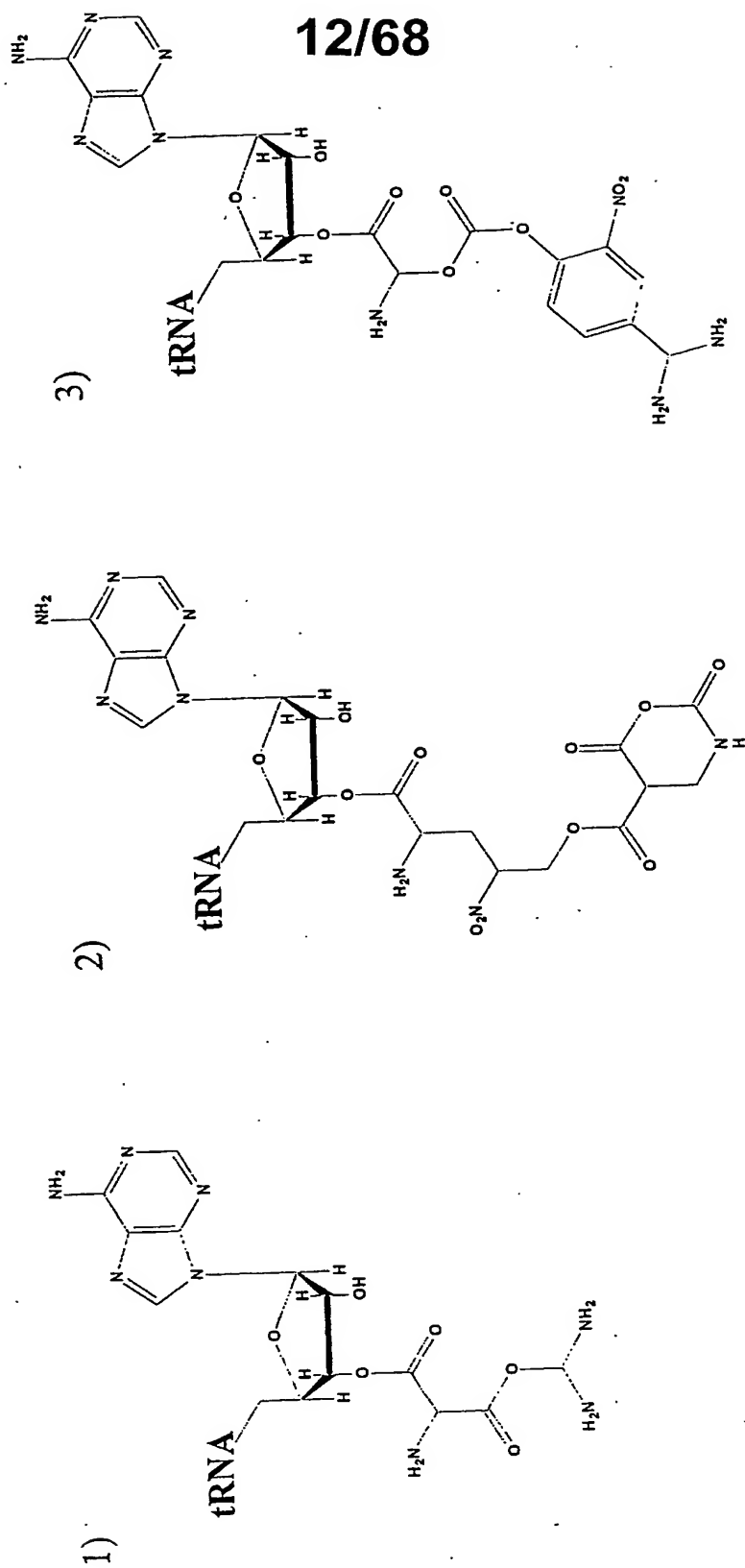
Example of a second building block

Fig. 4C

Examples of tRNAs charged with FE-AA units



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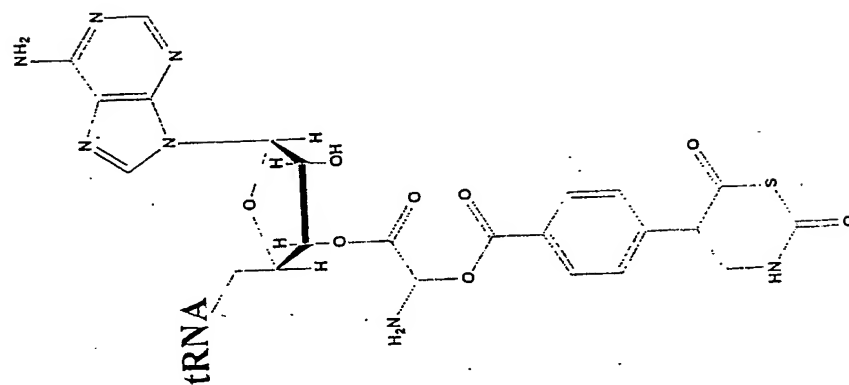
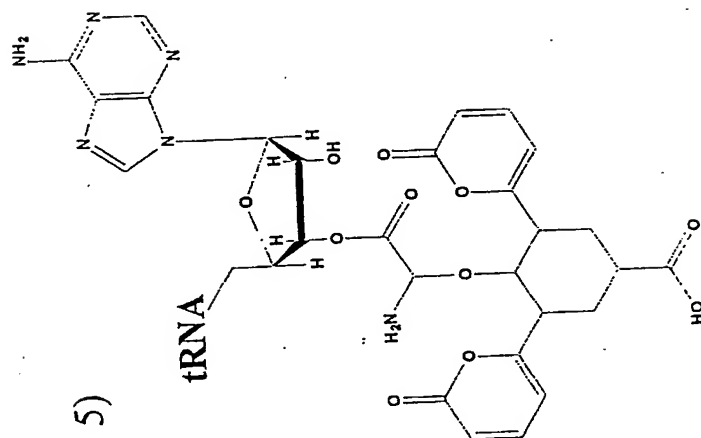
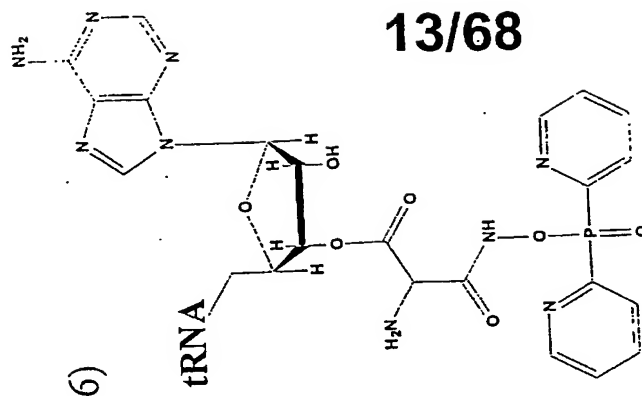


Fig. 4C, continued

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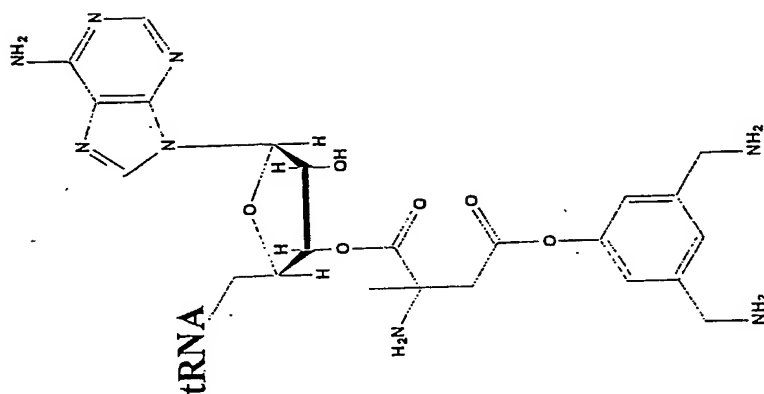
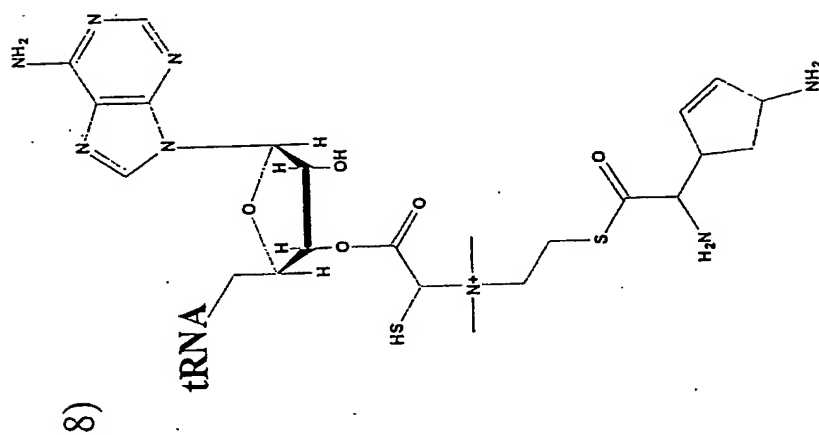
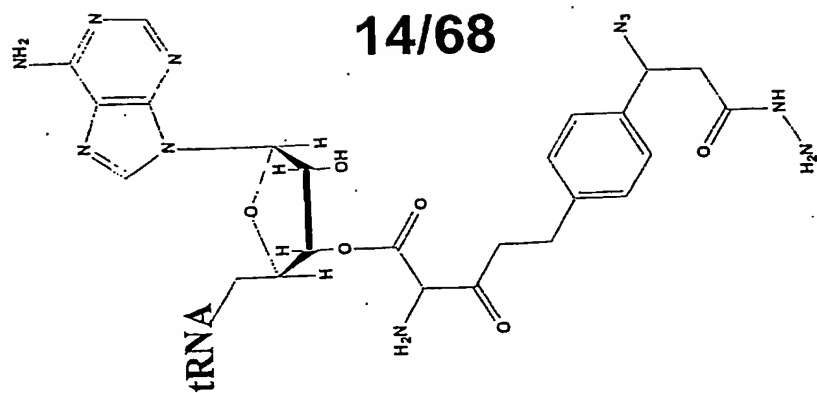


Fig. 4C, continued

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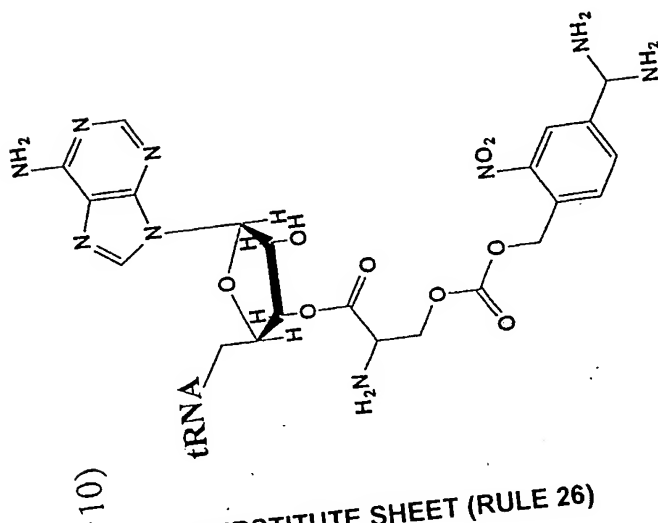
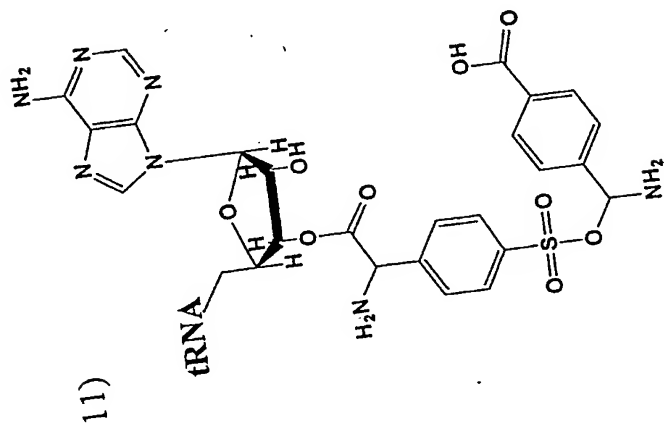
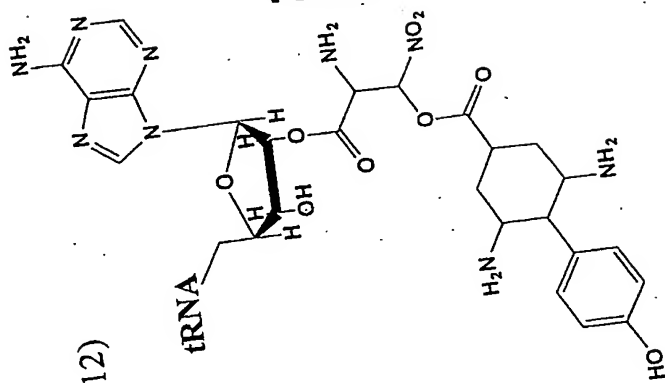


Fig. 4C, continued

Fig. 4C, continued

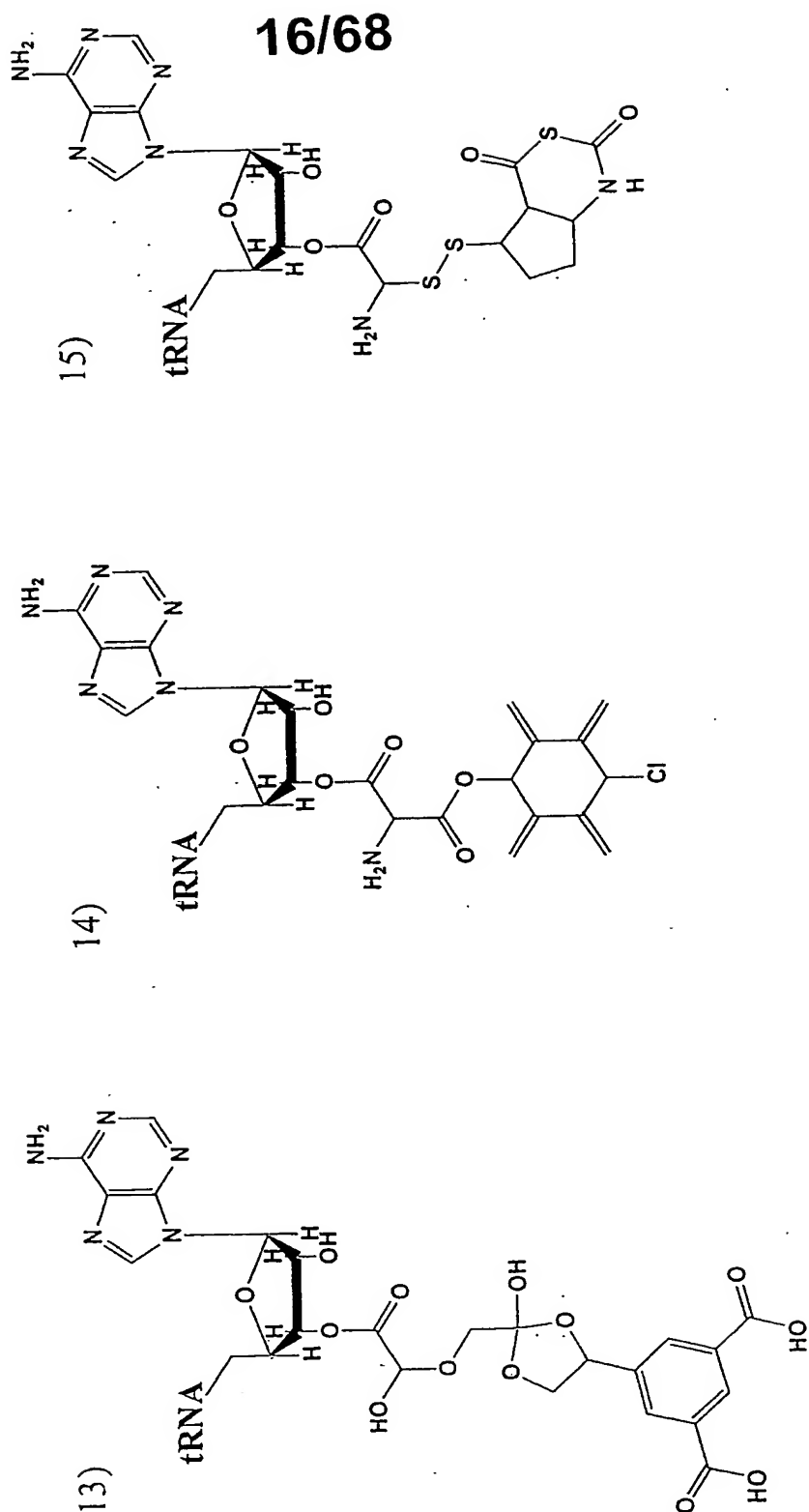
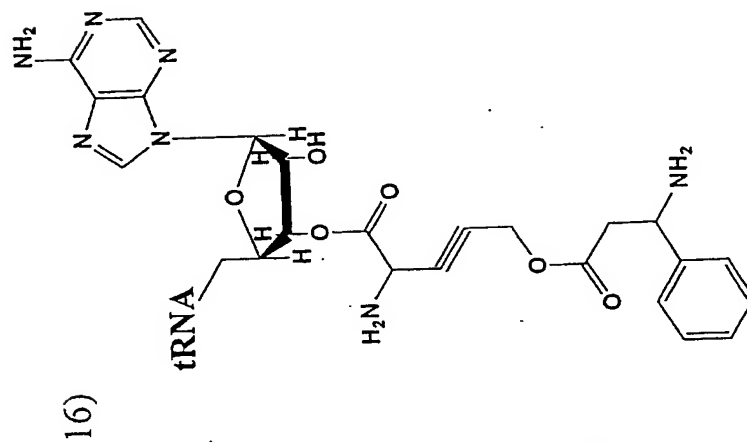
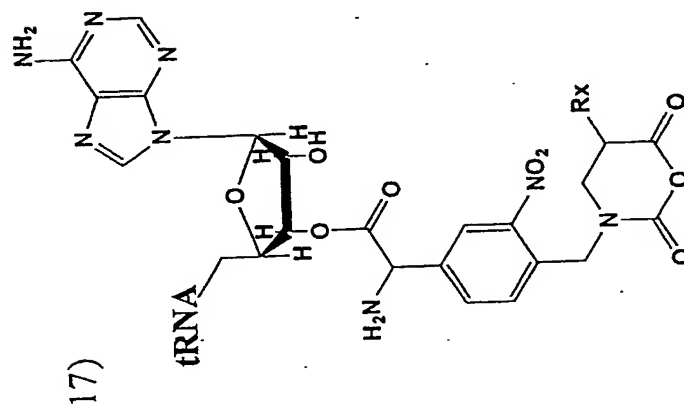
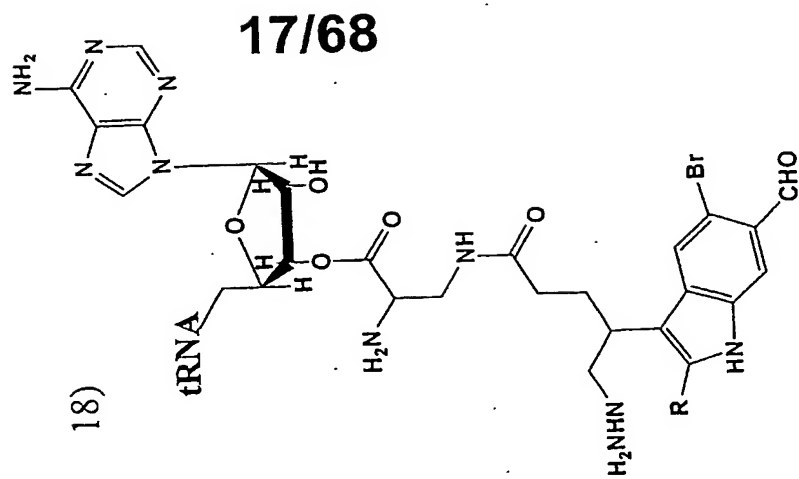


Fig. 4C, continued



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Fig. 5A

Enzymatic charging of tRNAs catalysed by amino acid tRNA synthetases

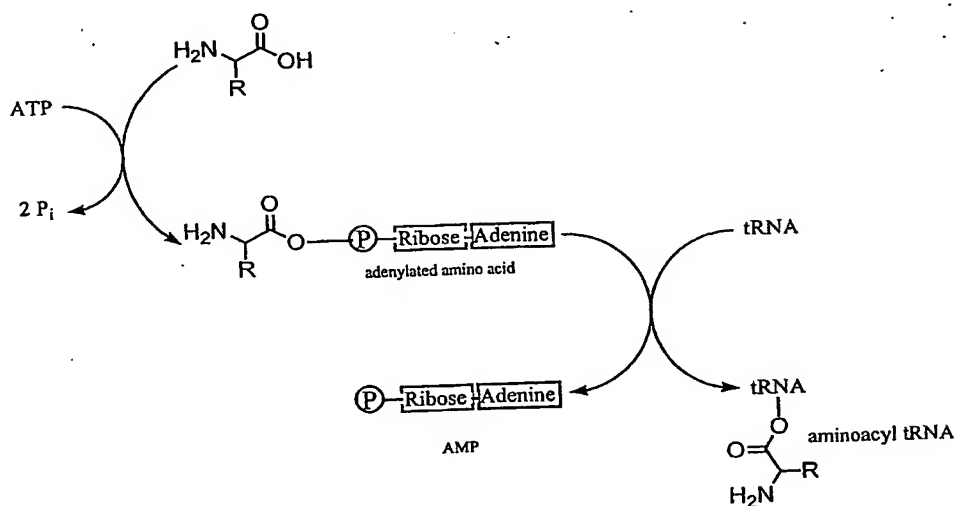
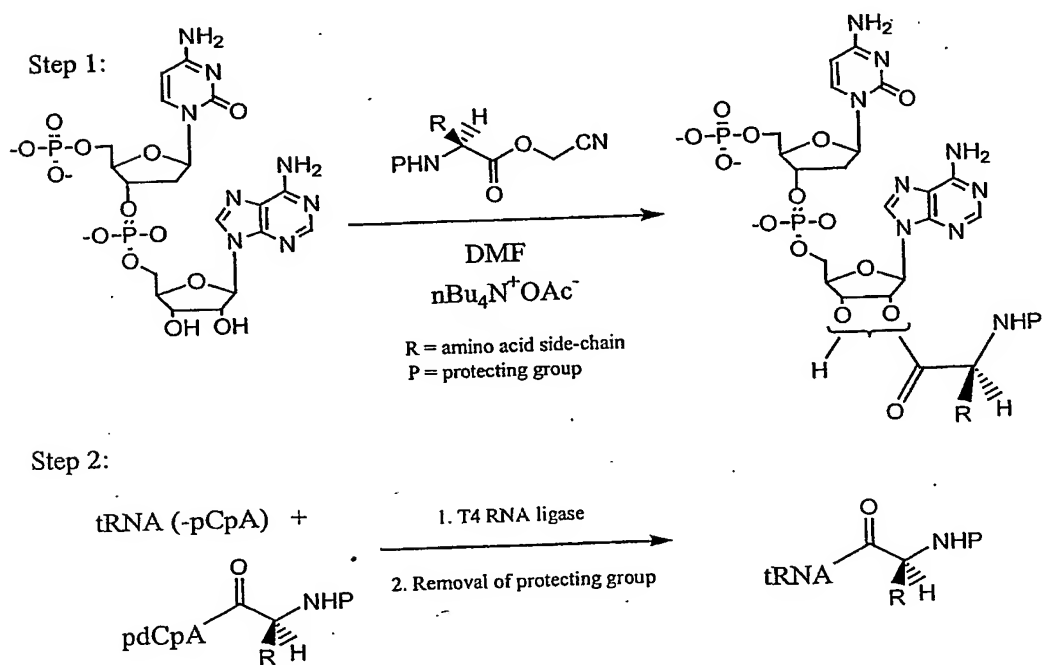


Fig. 5B

Chemical aminoacylation of tRNAs

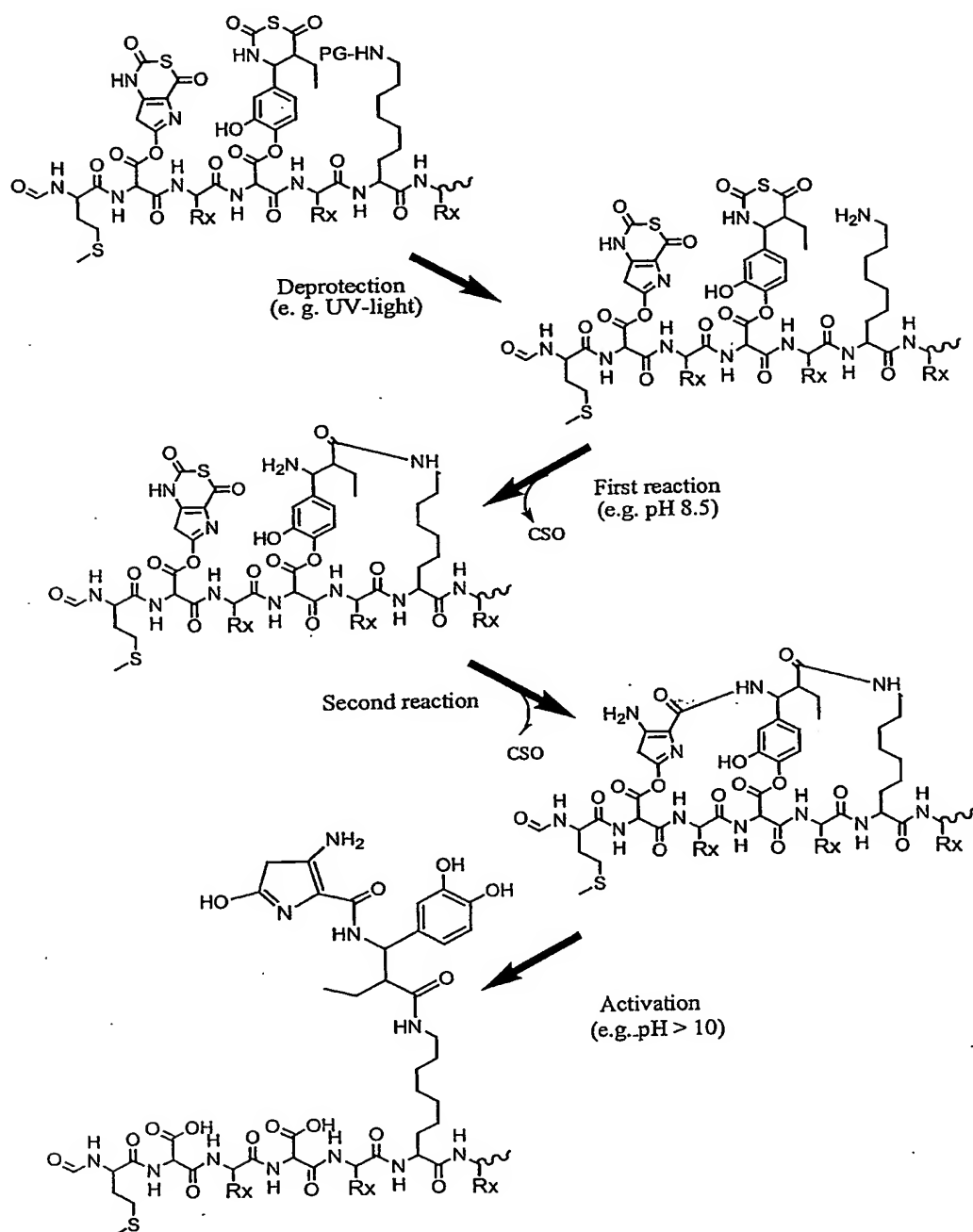


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Fig. 6

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**Bond formation between functional entities and
activation of the templated molecule**

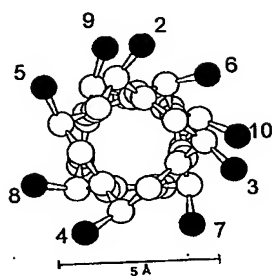


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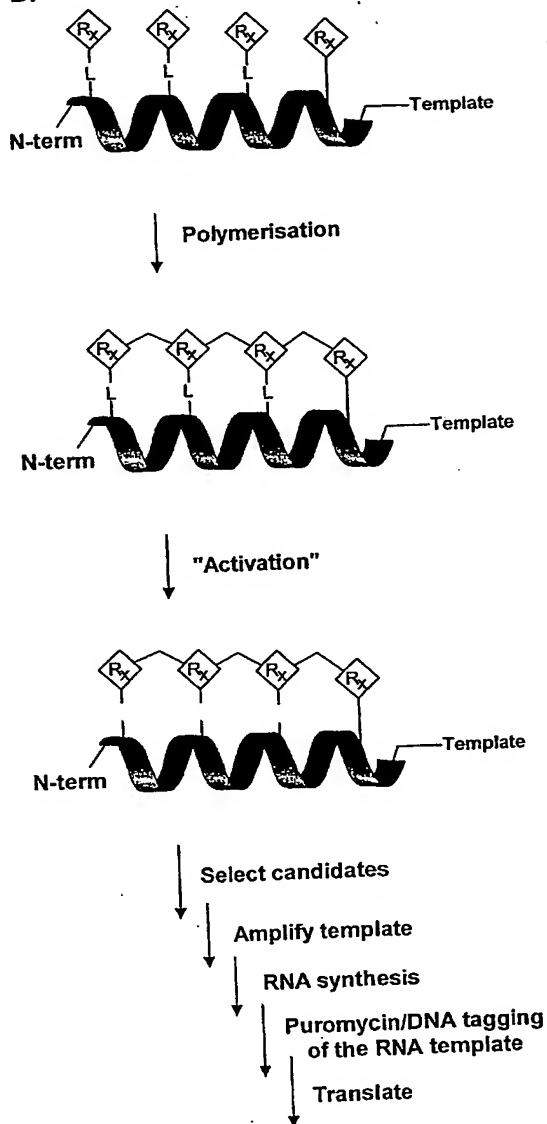
Fig. 7

alpha-helix display of functional entities

A:



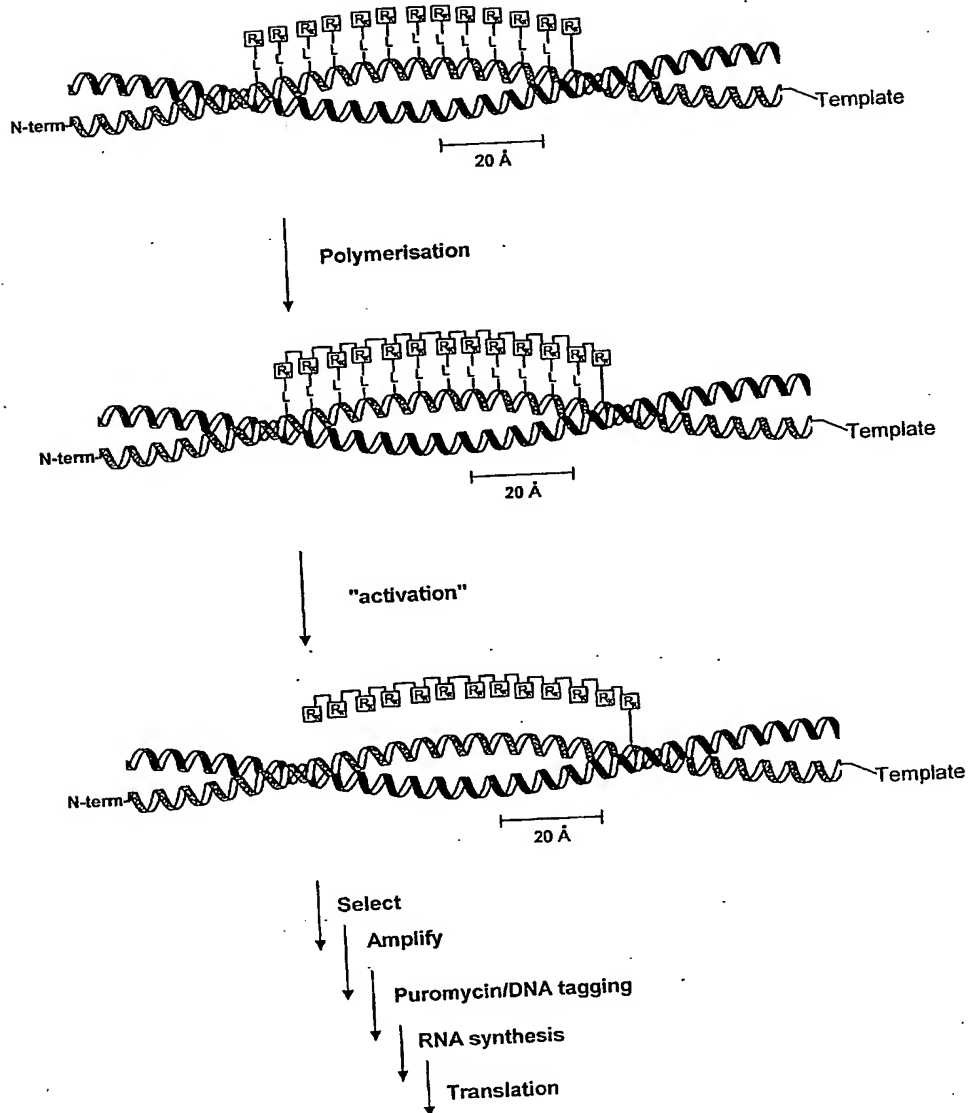
B:



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Fig. 8

Coiled-coil display of functional entities



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Fig. 9

. Display of functional entities by a collagen-like triple helix structure

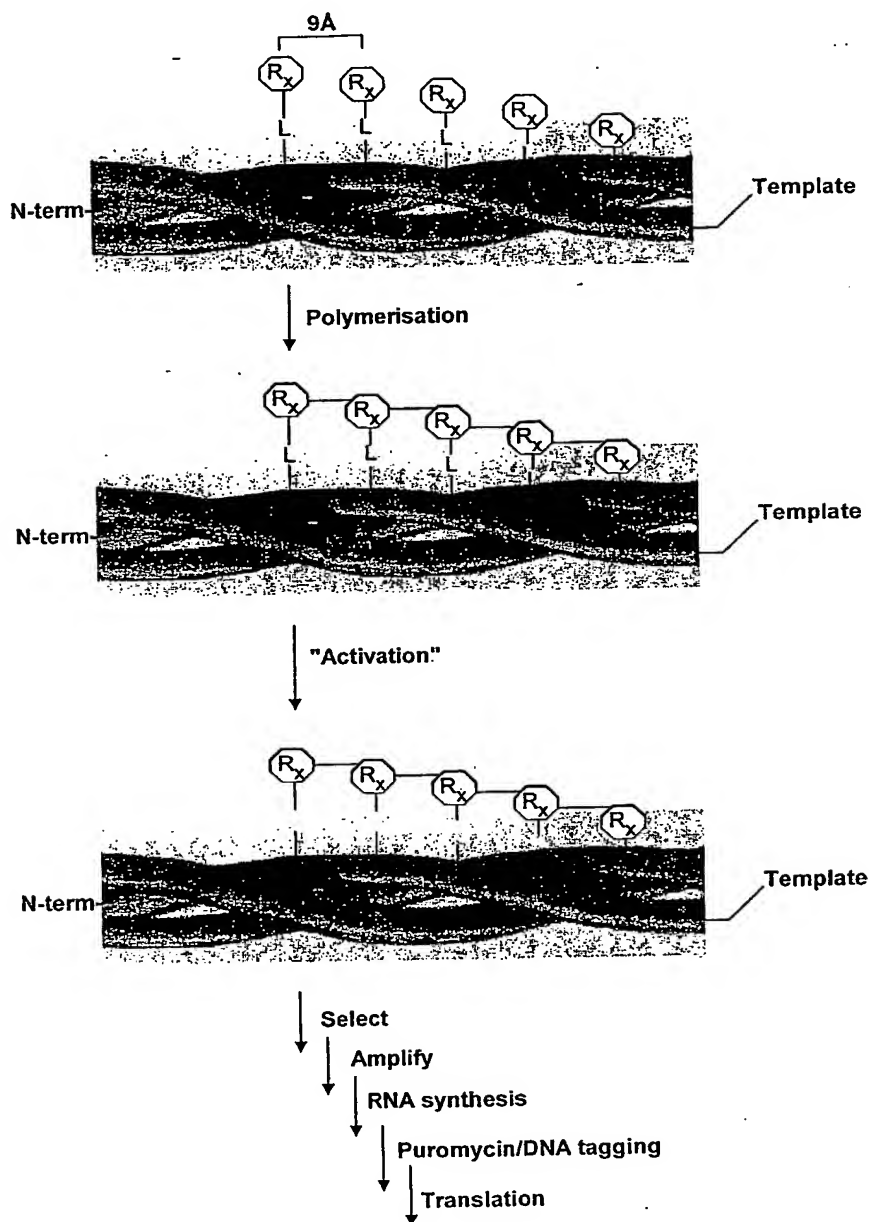
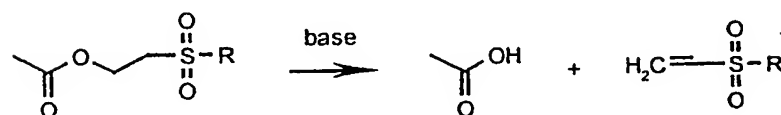
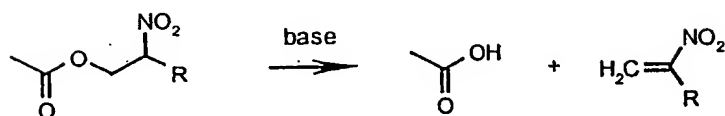
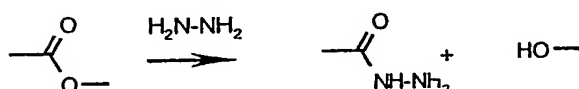
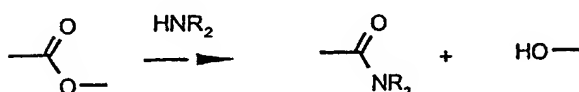
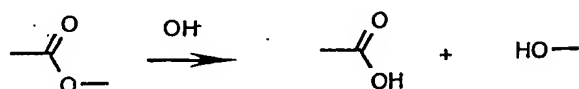


Fig. 10

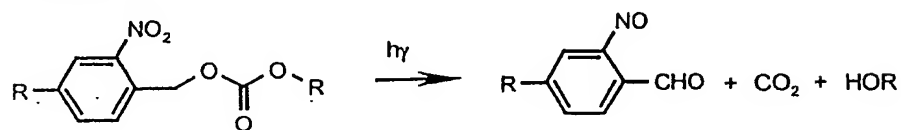
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Cleavable linkers and protection groups, cleaving agents and cleavage products.

A. Base (nucleophilic) cleavage.



B. Photocleavage

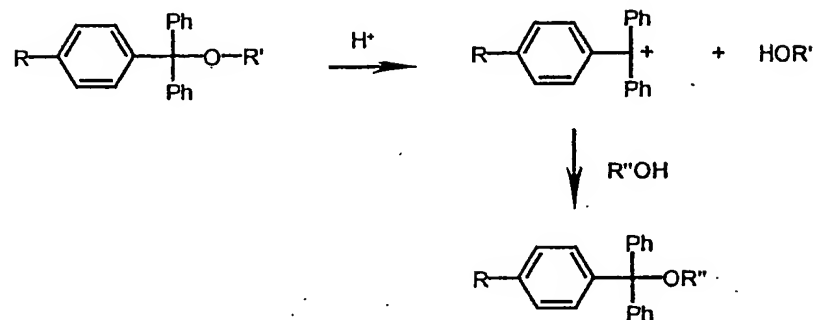
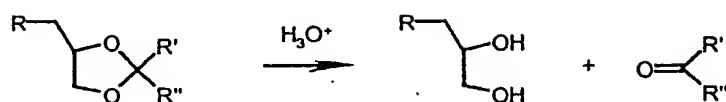
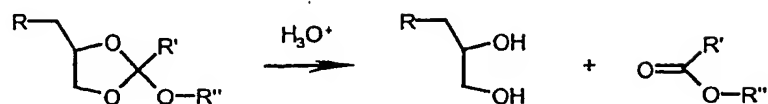


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Fig. 10, continued

C. Acid cleavage



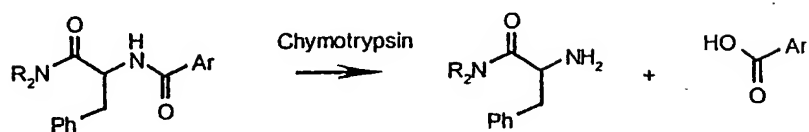
D. Catalytic cleavage.



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Fig. 10, continued

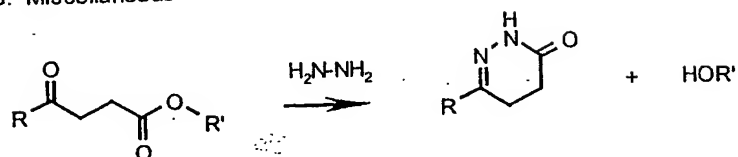
E. Enzymatic cleavage.



F. Cleavage by temperature increase.



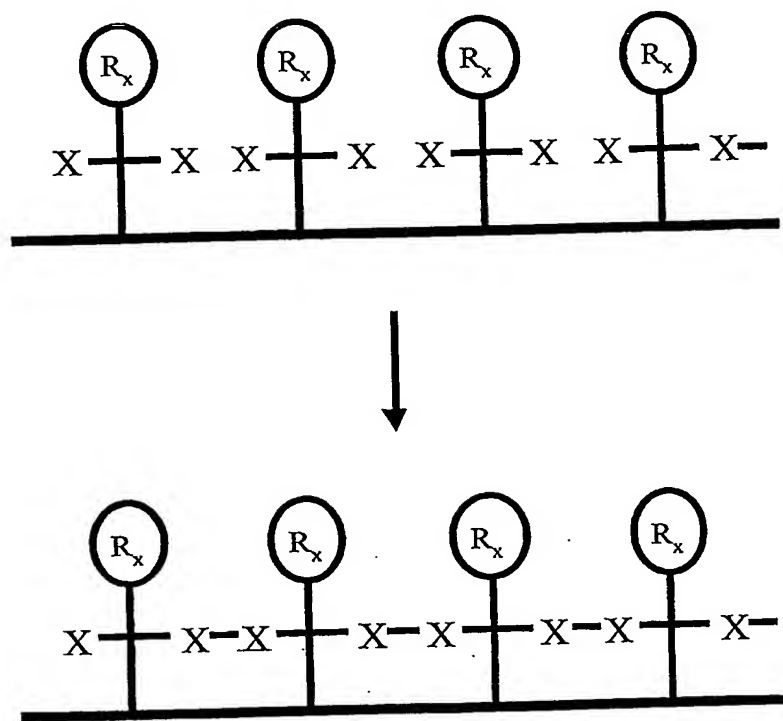
G. Miscellaneous



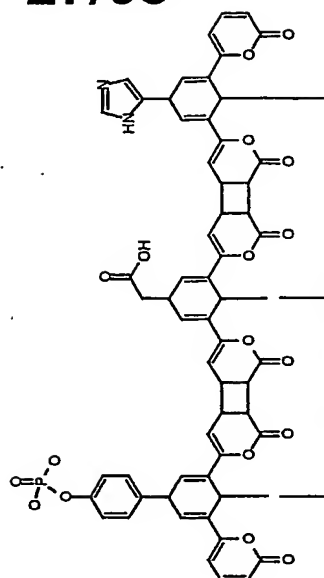
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Fig. 11

Polymerization by reaction between neighboring reactive groups.



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$h\nu$ → activate →

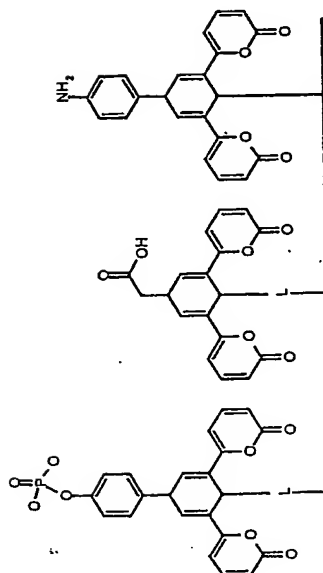
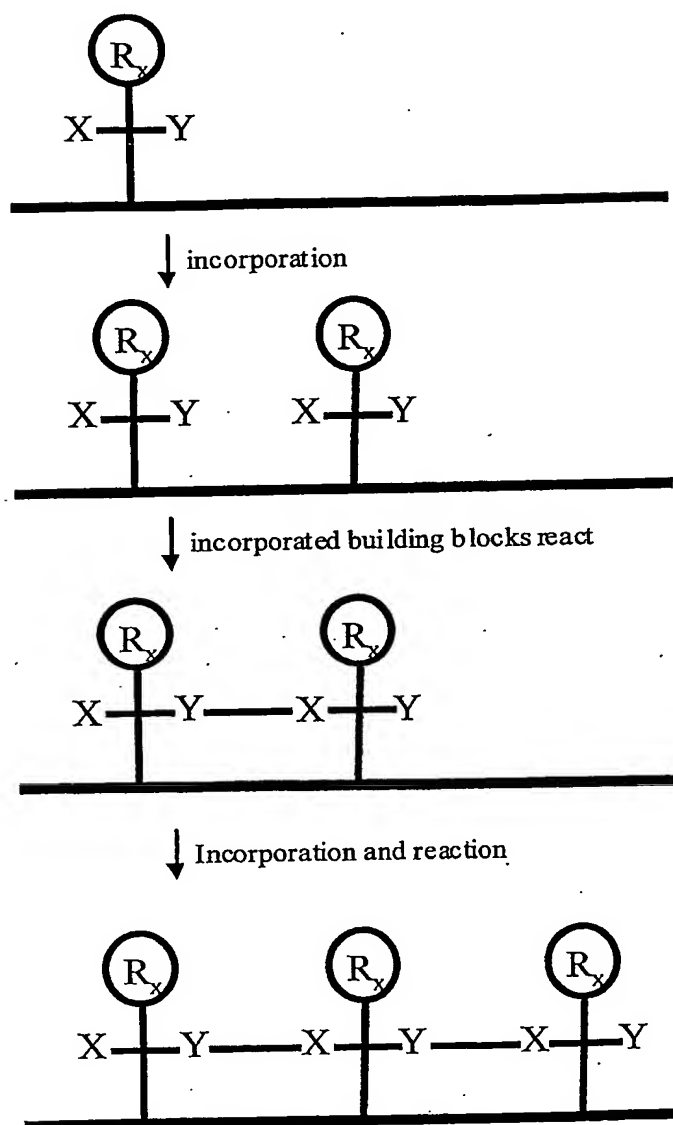


Fig. 11, continued

Ex. 1. Coumarin-based polymerization

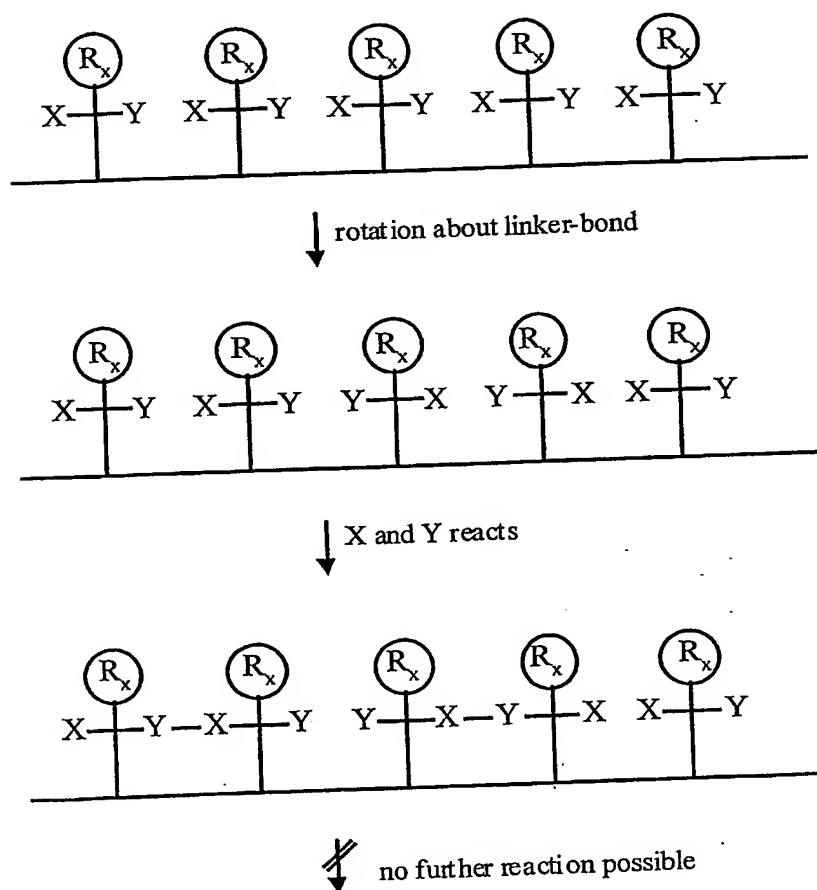
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Fig 12. Polymerization between neighboring non-identical reactive groups.



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Fig. 13. Cluster formation in the absence of directional polymerization.



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Fig 14. Zipping-polymerization and simultaneous activation.

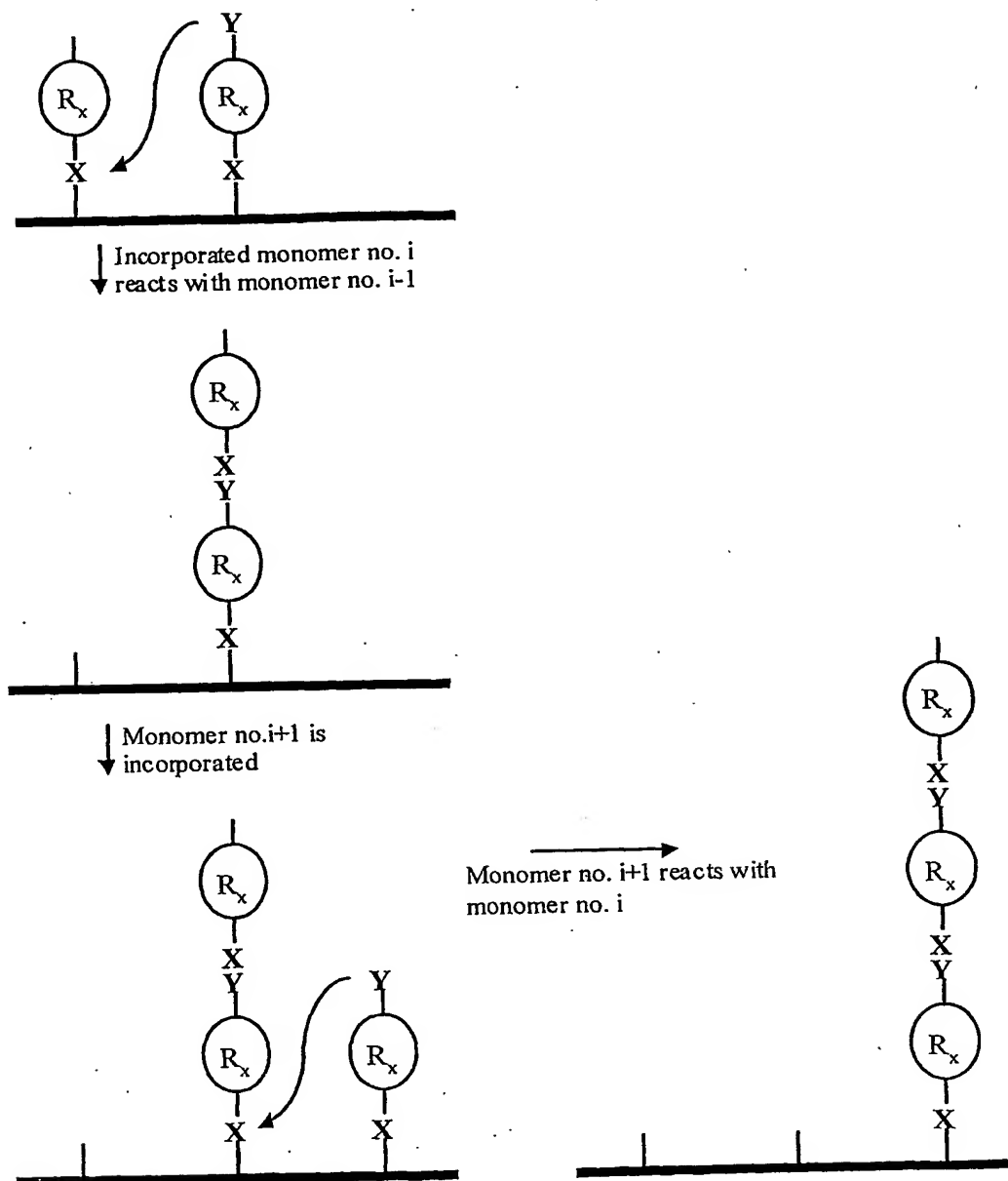
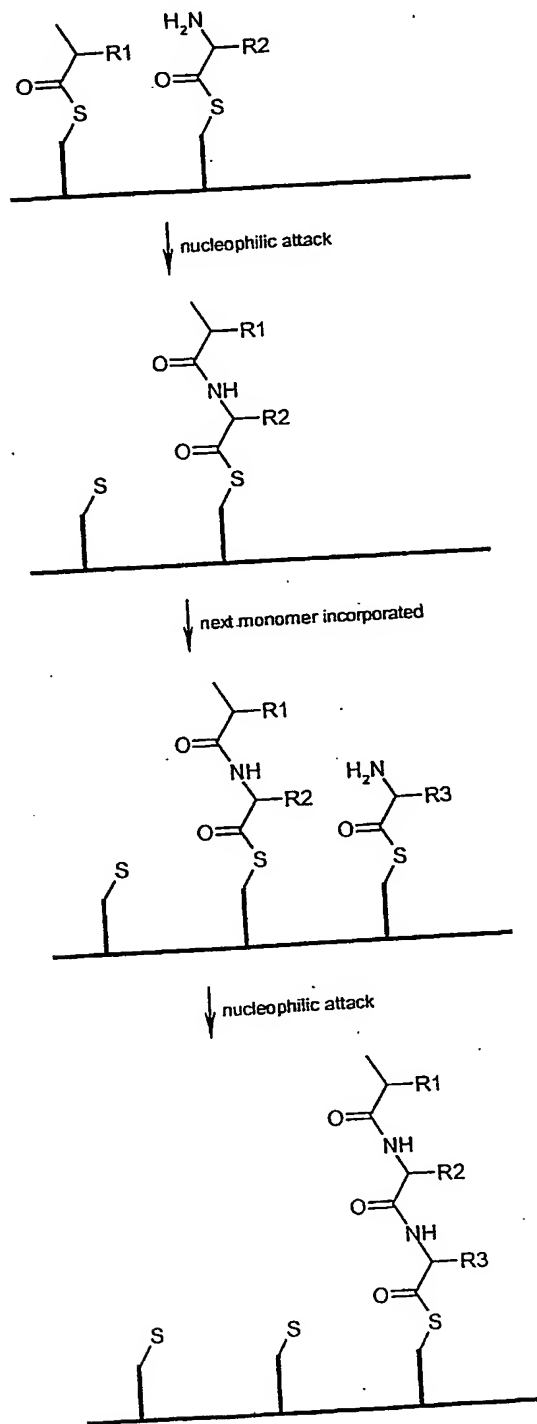


Fig. 14, continued **31/68**

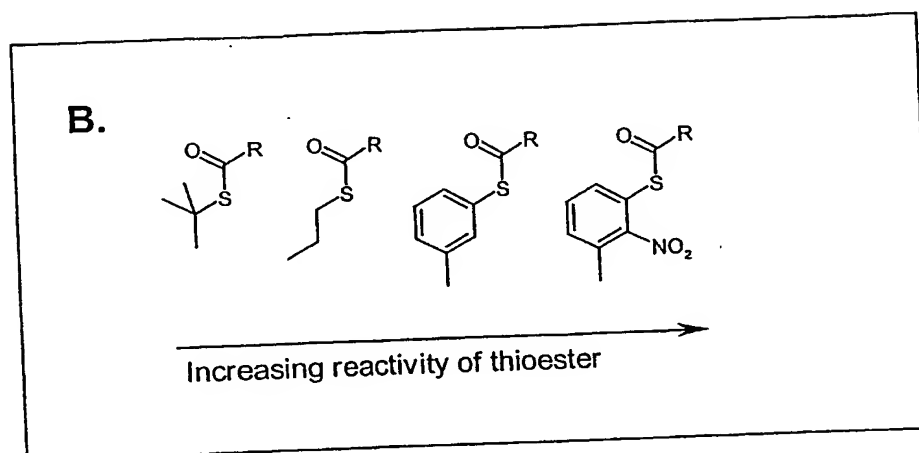
Example 1. Polymerization and activation (thioesters)

A.

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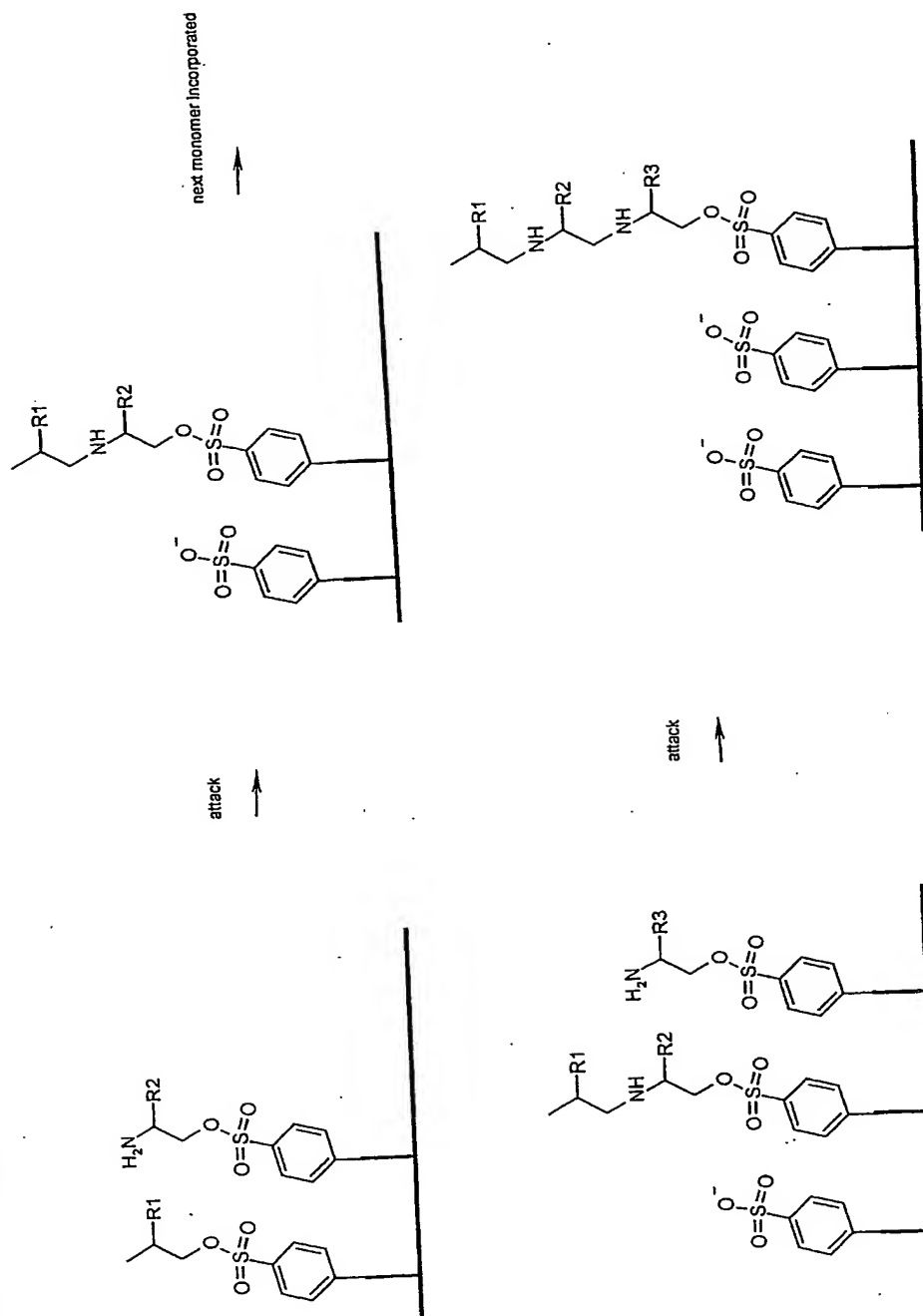
Fig. 14, continued



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Fig. 14, continued

Example 2. Polyamine formation and activation



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Fig. 15

"Fill-in" polymerization (symmetric XX monomers).

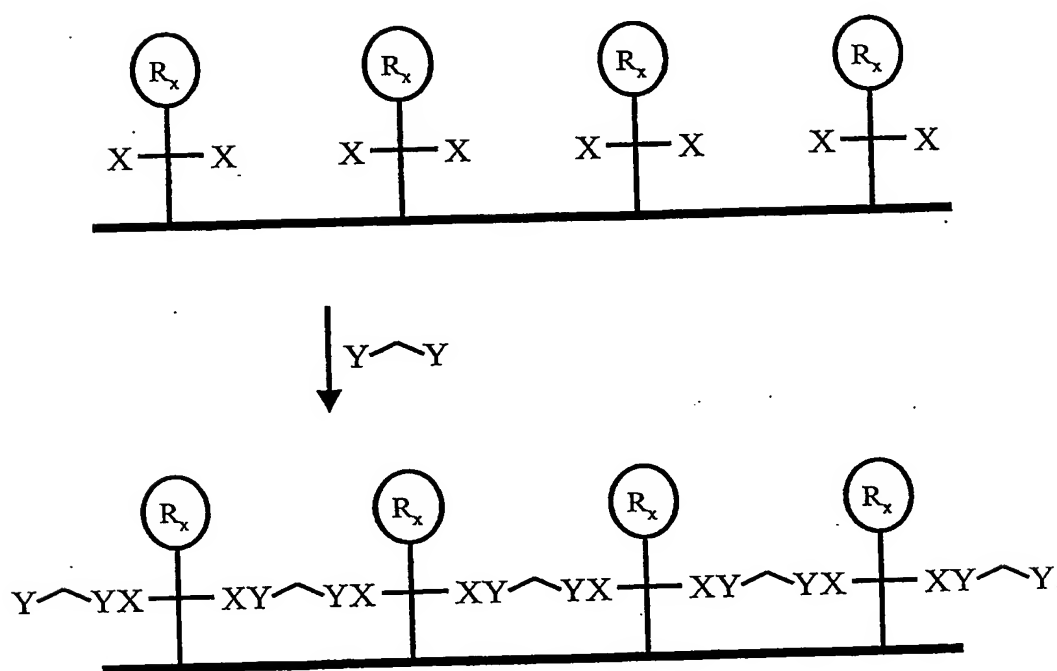
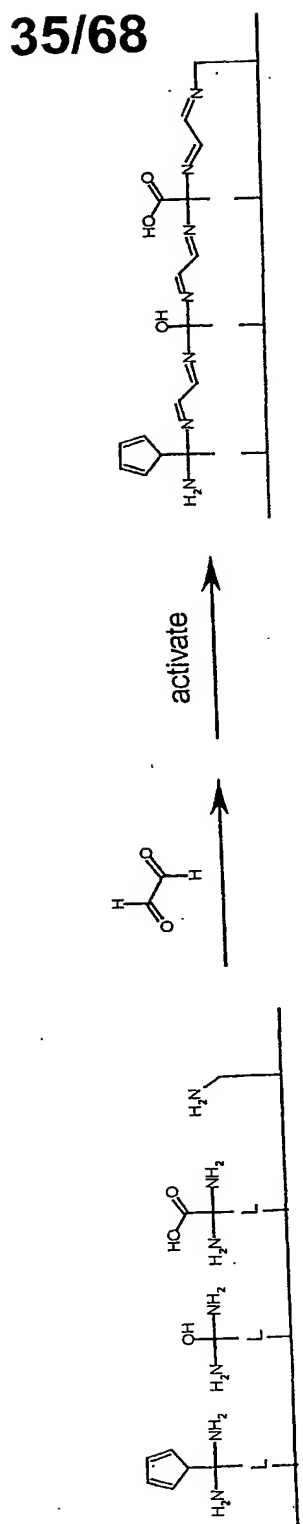


Fig. 15, continued
Example 1. Poly-imine formation by fill-in polymerization



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Fig. 15, continued

Example 2. Polyamide formation

A.

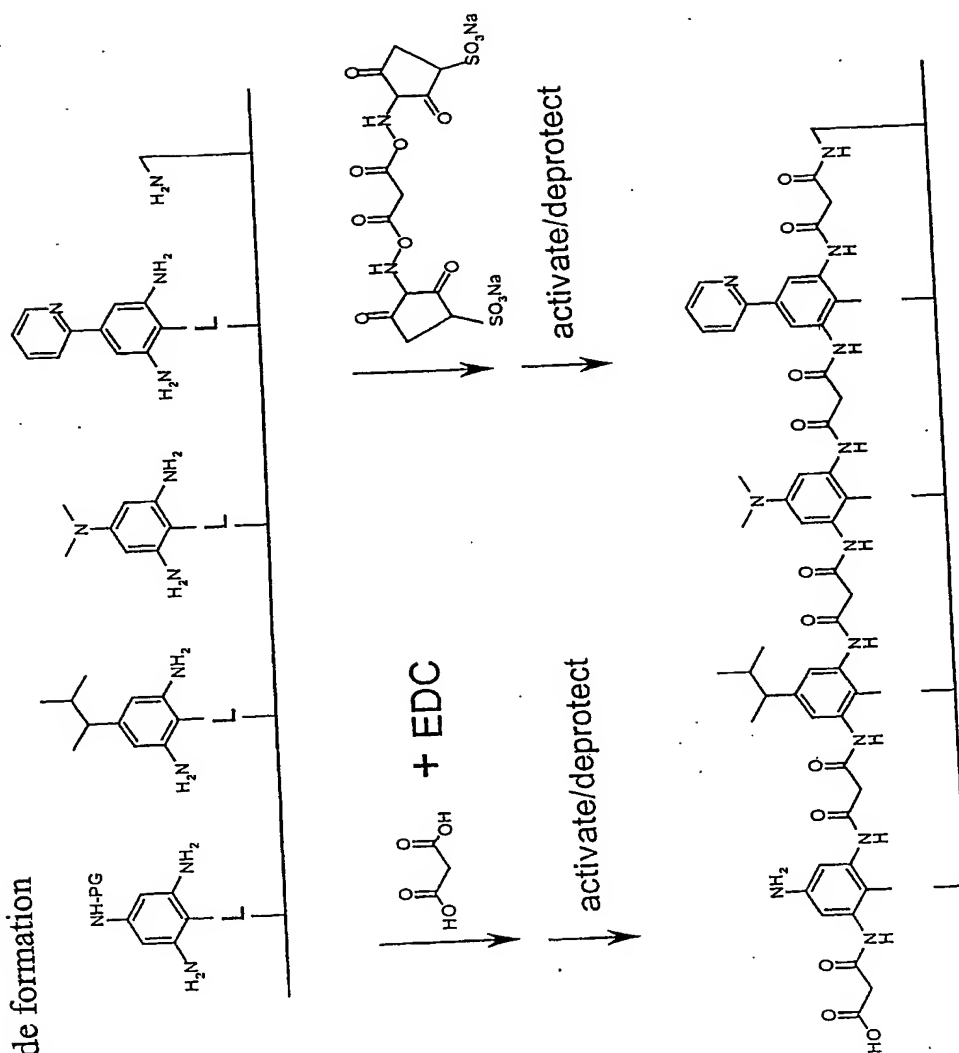
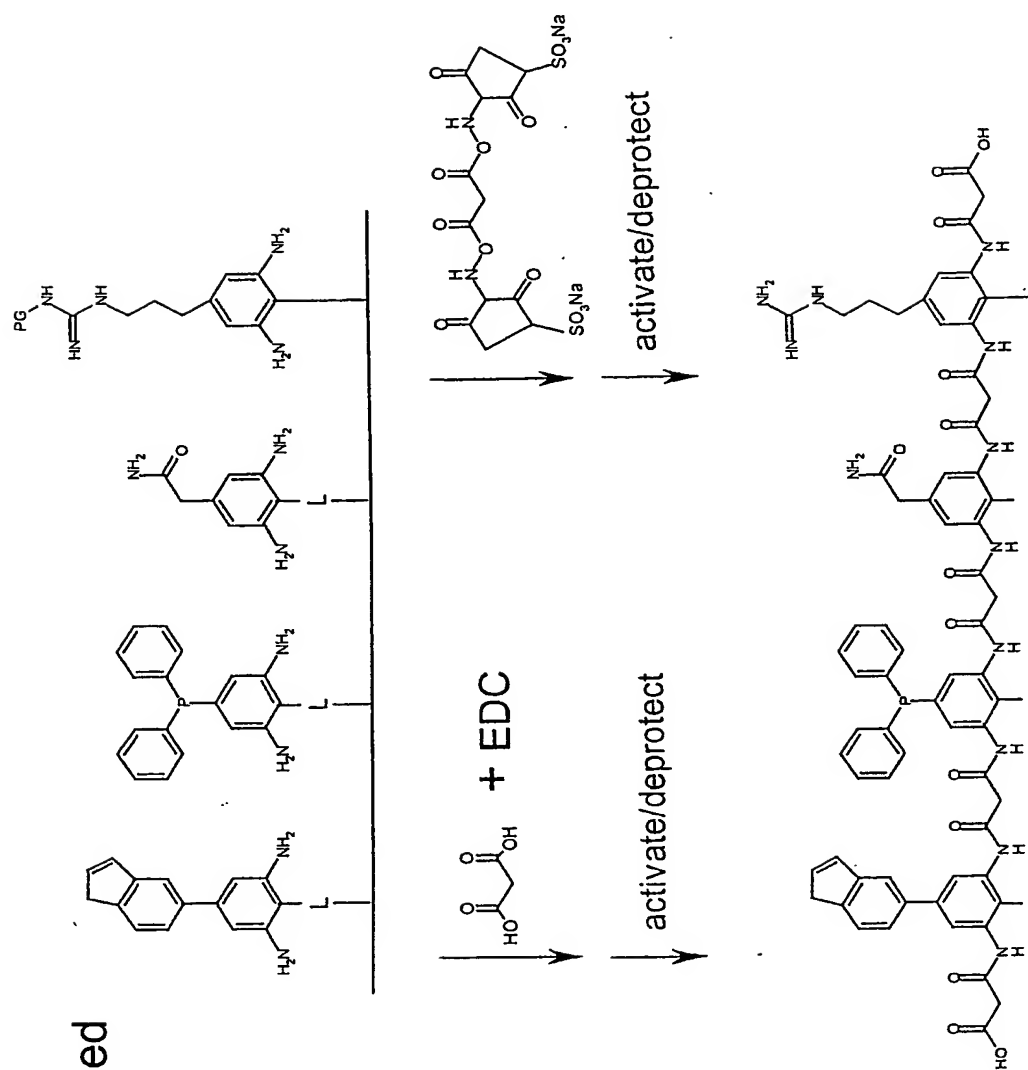


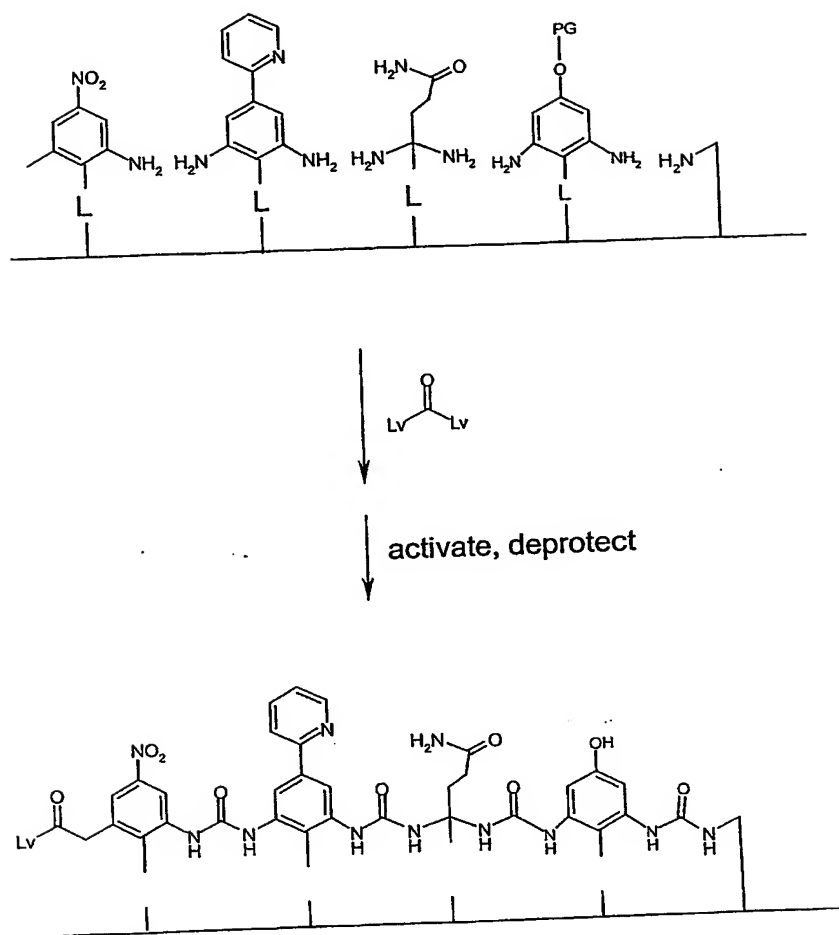
Fig. 15, continued



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Fig. 15, continued

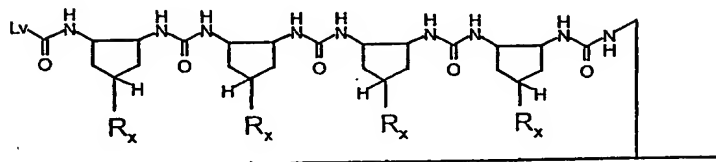
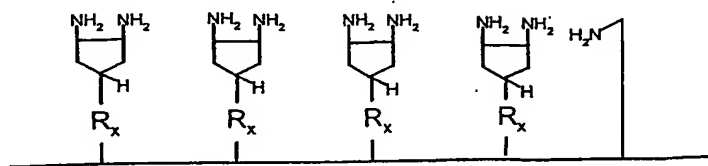
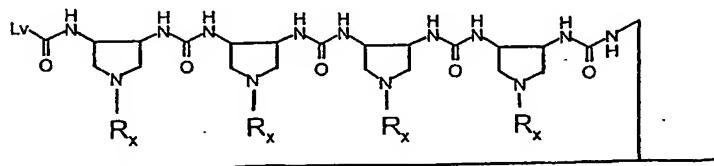
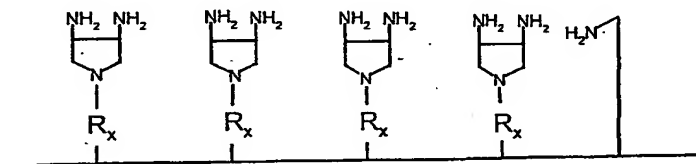
Example 3. Polyurea formation



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Fig. 15, continued **39/68**

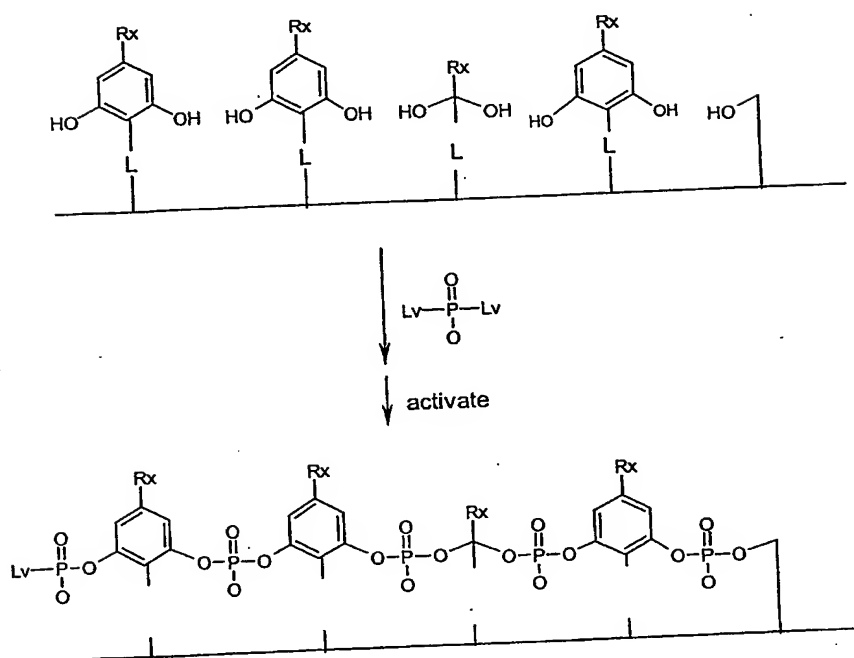
Example 4. Chiral and achiral polyamide backbone formation

A.**B.**

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Fig. 15, continued

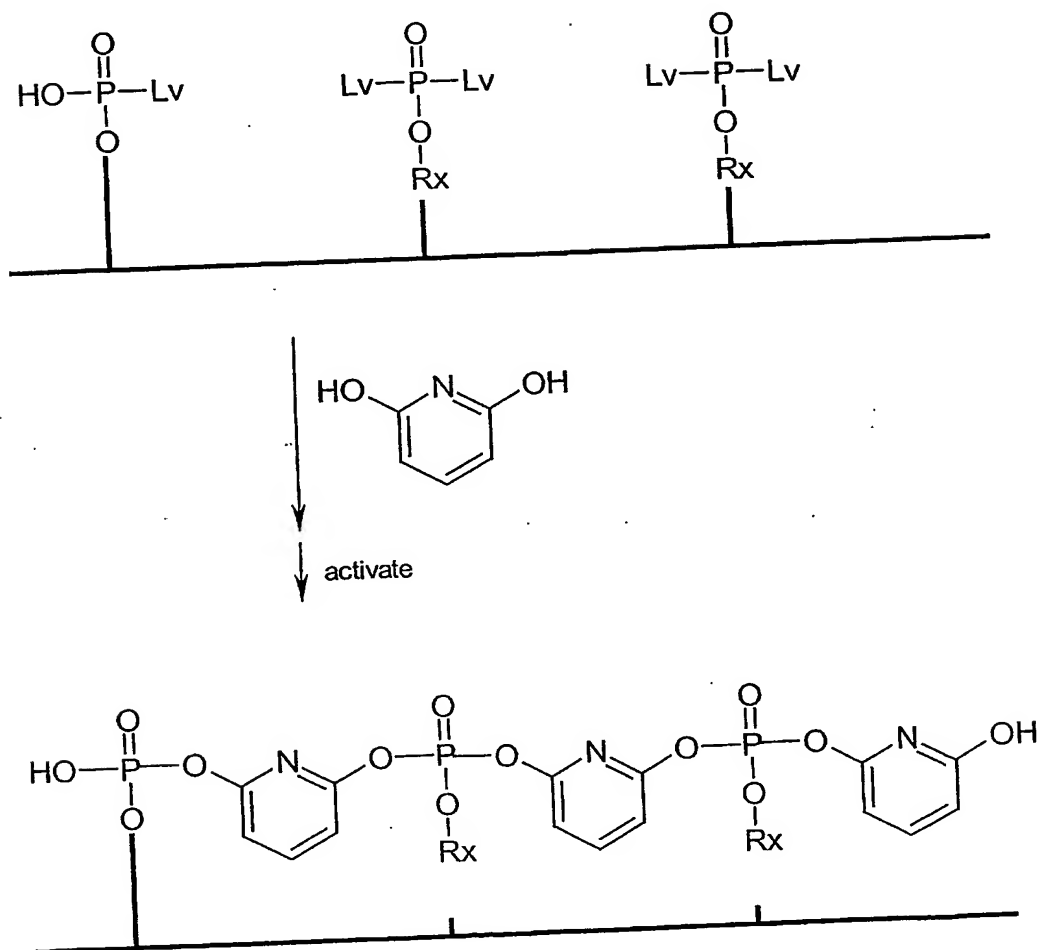
Example 5. Polyphosphodiester formation



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Fig. 15, continued

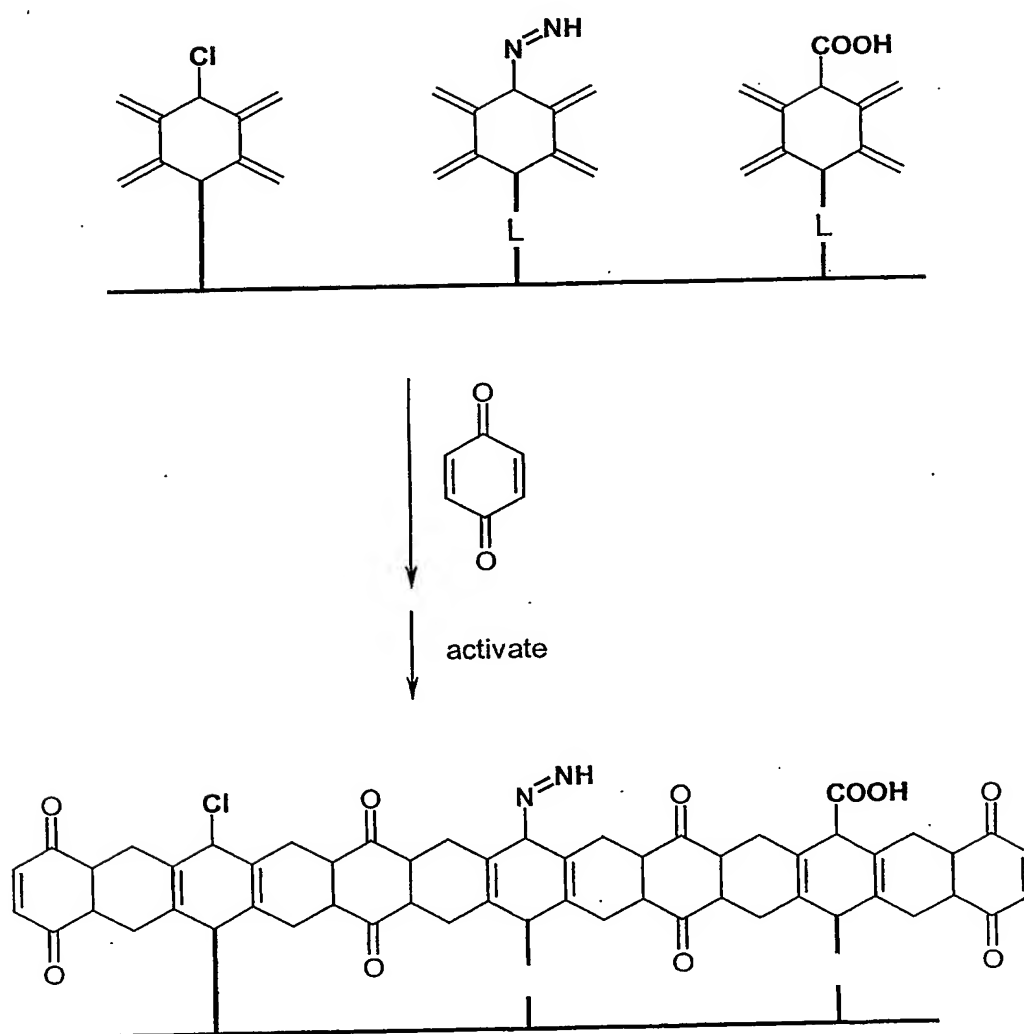
Example 6. Polyphosphodiester formation with one reactive group in each monomer building



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Fig. 15, continued

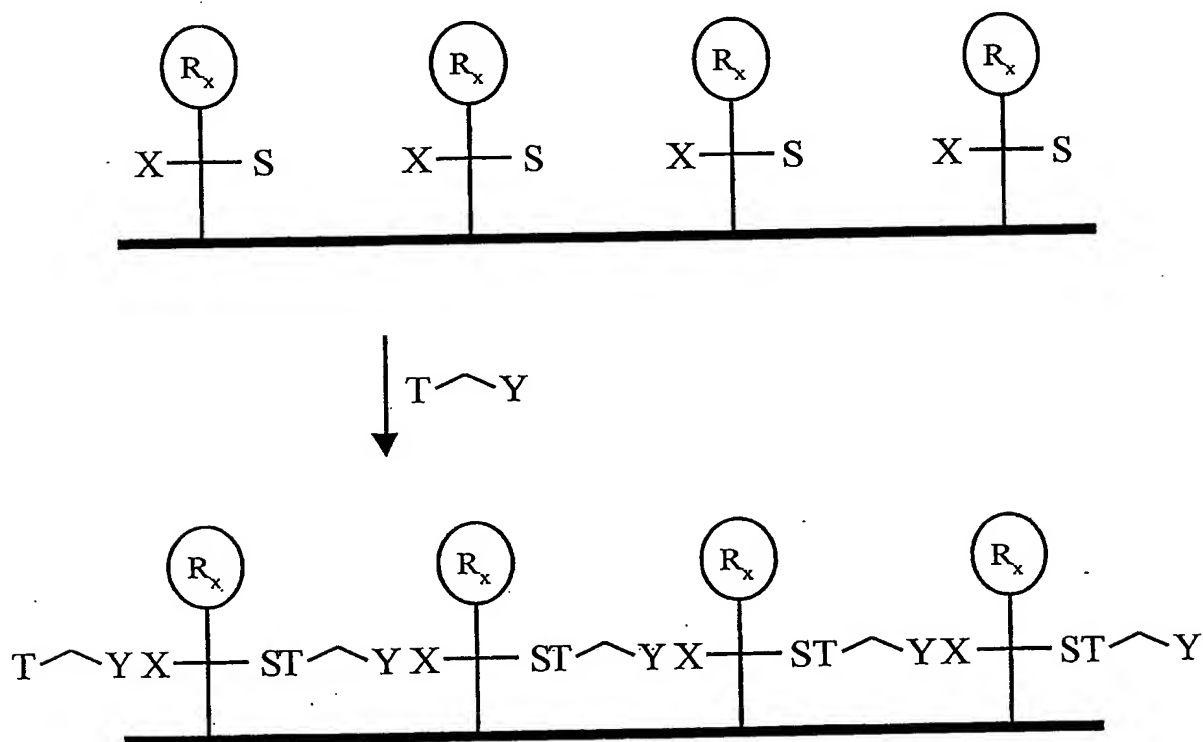
Example 7. Pericyclic, "fill-in" polymerization



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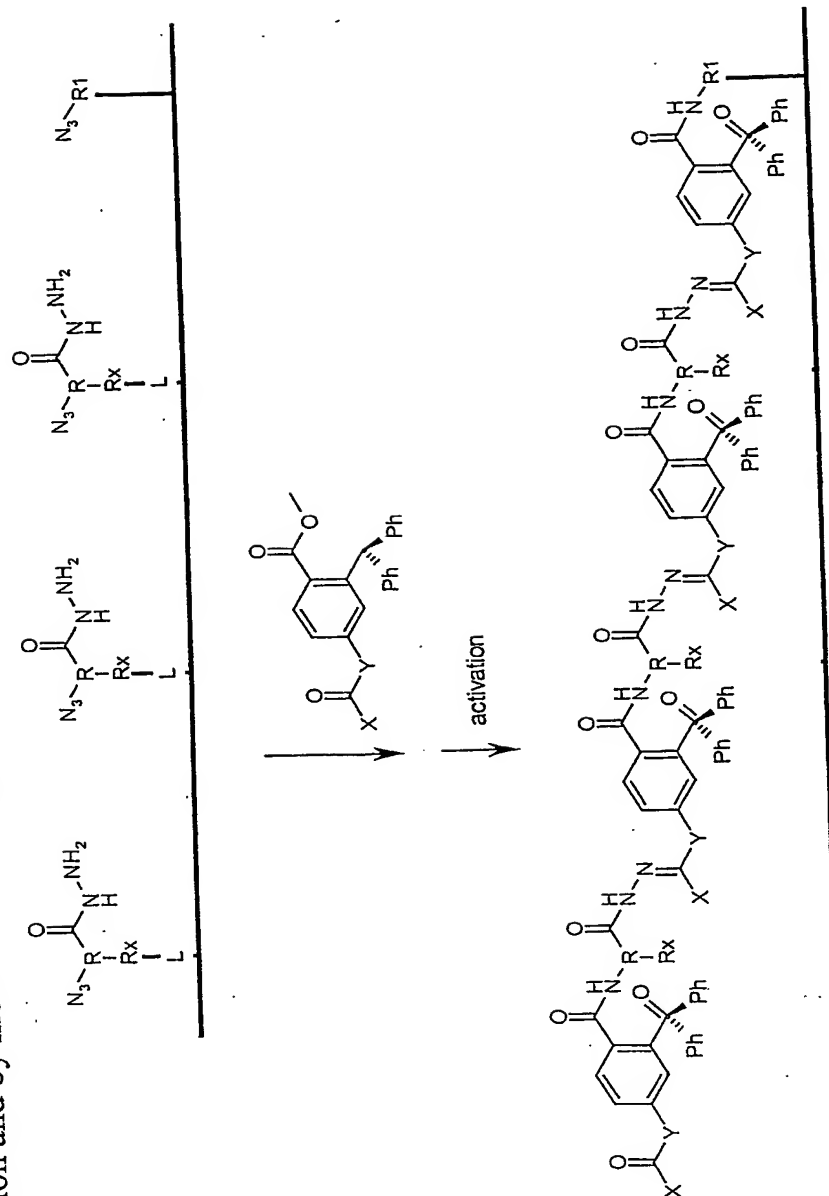
Fig. 16. "Fill-in" polymerization (asymmetric XS monomers).



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Fig. 16, continued

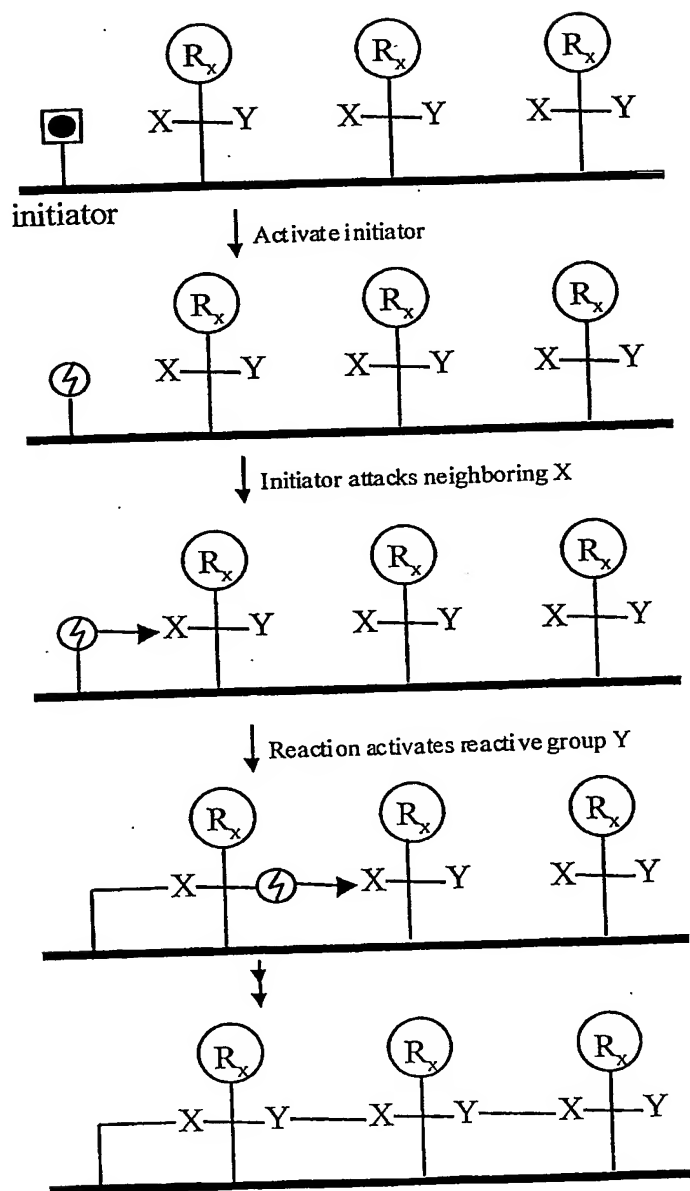
Example 1. Fill-in polymerization by ketone-hydrazide reaction and by modified Staudinger ligation



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Fig. 17

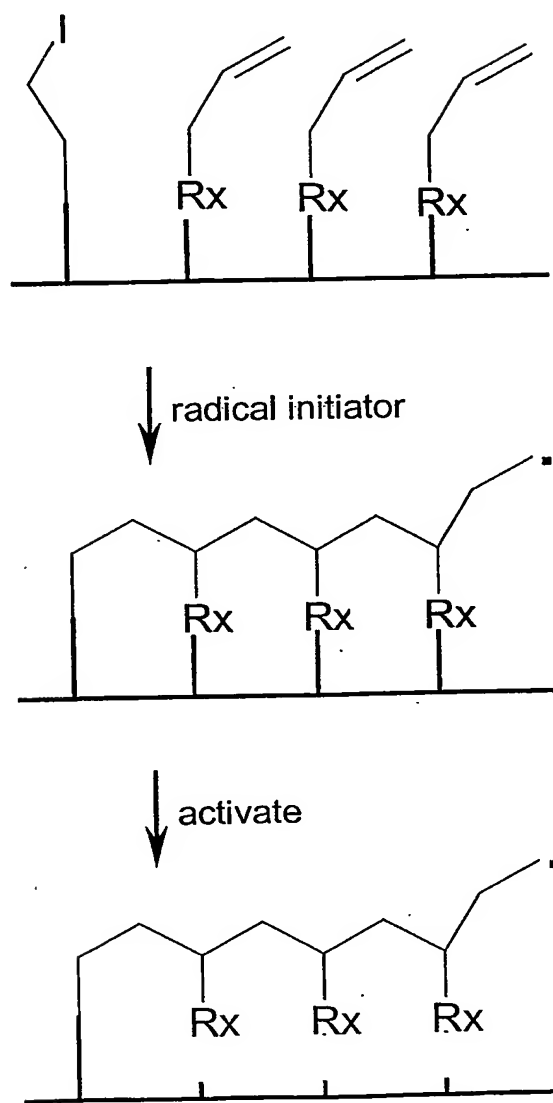
"Zipping" polymerization



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Fig. 17, continued

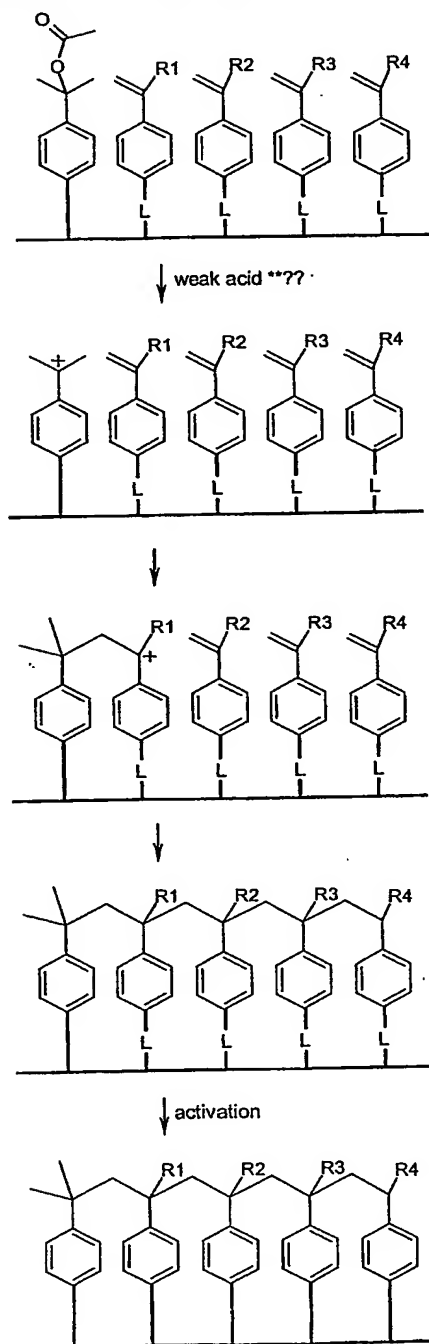
Example 1. Radical polymerization



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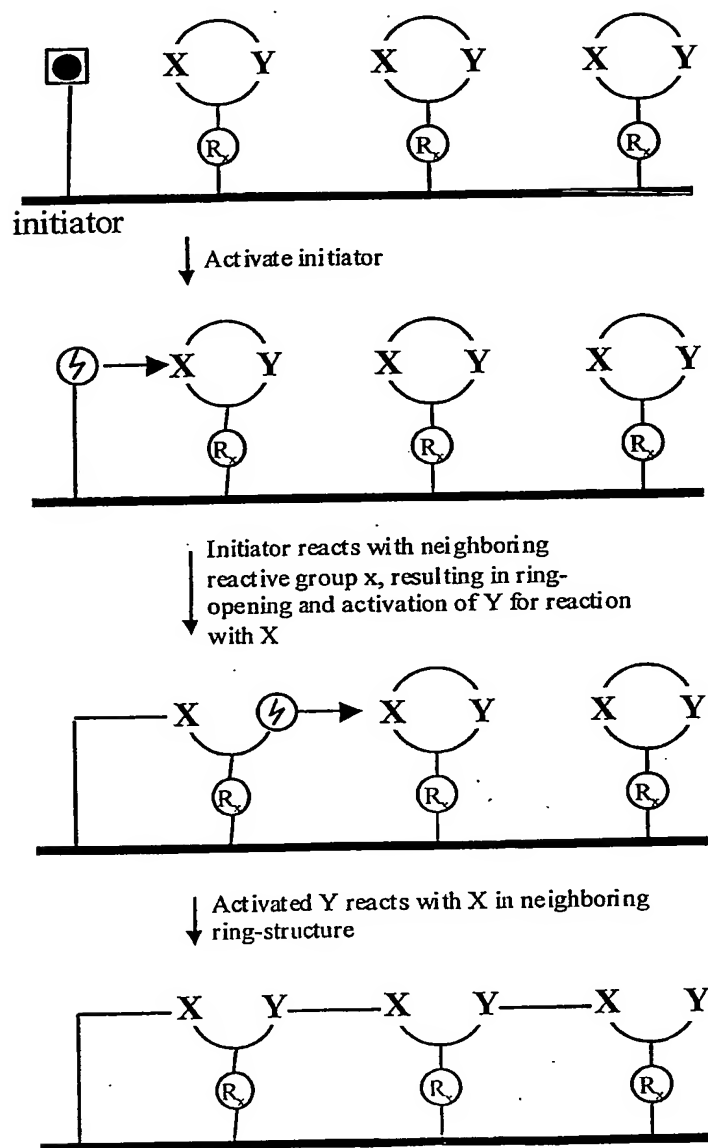
Fig. 17, continued. Example 2. Cationic polymerization



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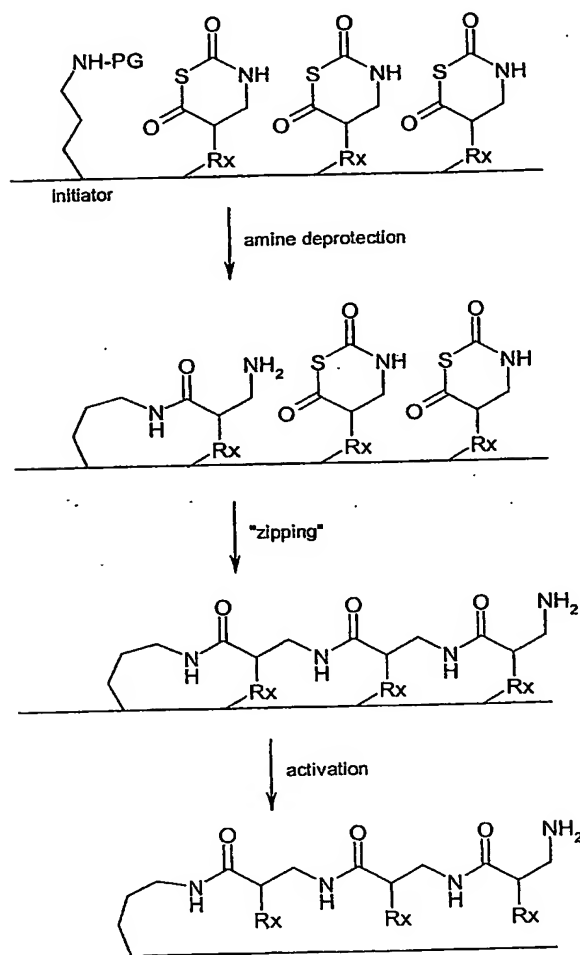
Fig. 18. Zipping polymerization by ring opening.



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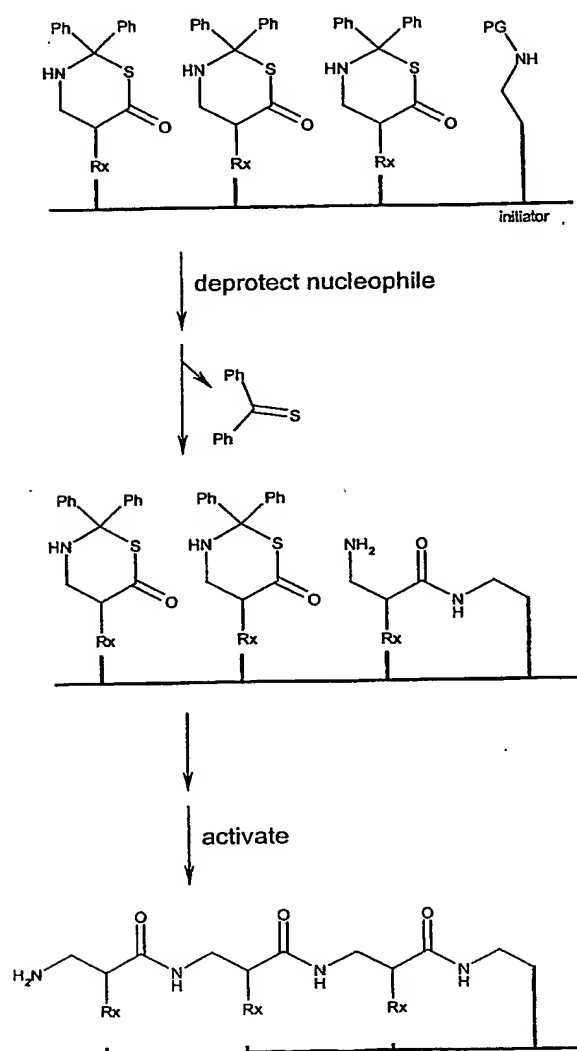
Fig. 18, continued. Example 1.

"Zipping" polymerization of N-thiocarboxyanhydrides, to form β -peptides.



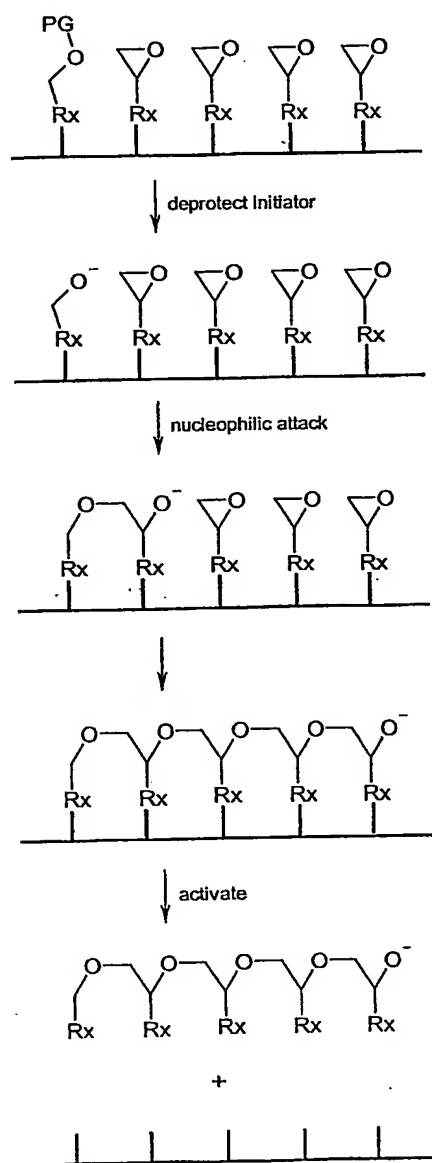
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Fig. 18, continued. Example 2. "Zipping"
polymerization of 2,2-diphenylthiazinanone units
to form β -peptides.



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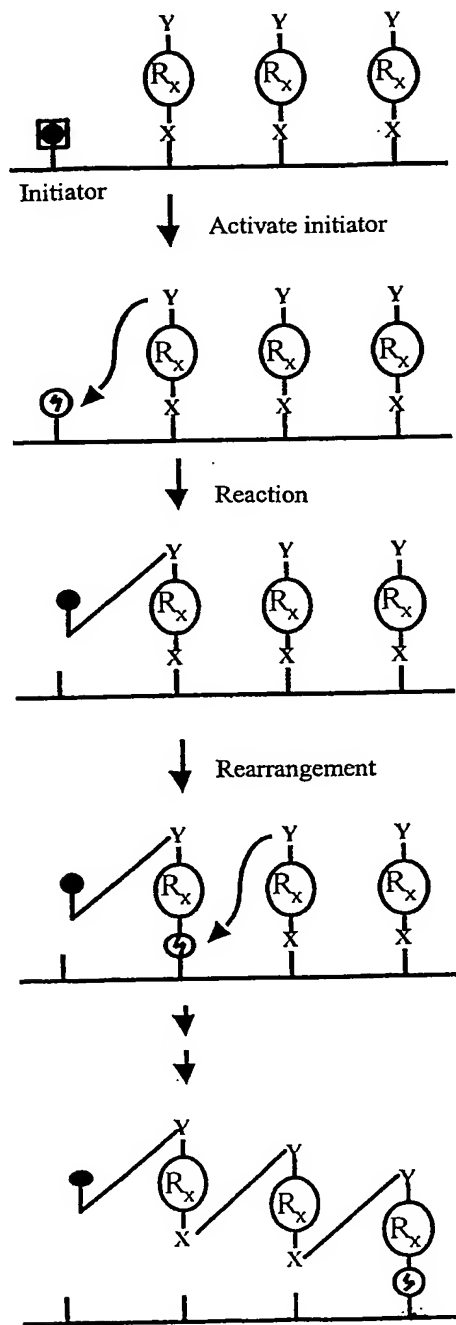
Fig. 18, continued. Example 3. Polyether formation by ring-opening polymerization.



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Fig. 19

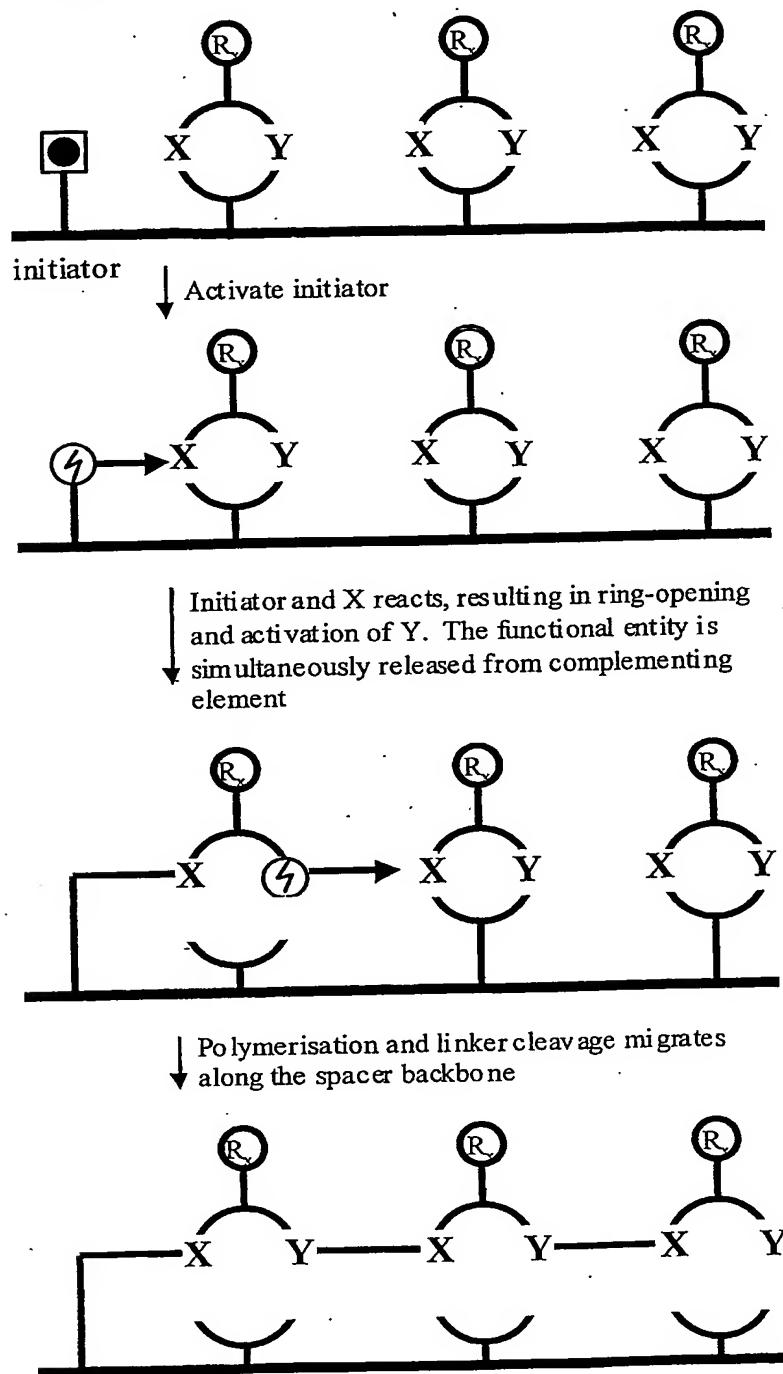
Zipping-polymerization and activation by rearrangement.



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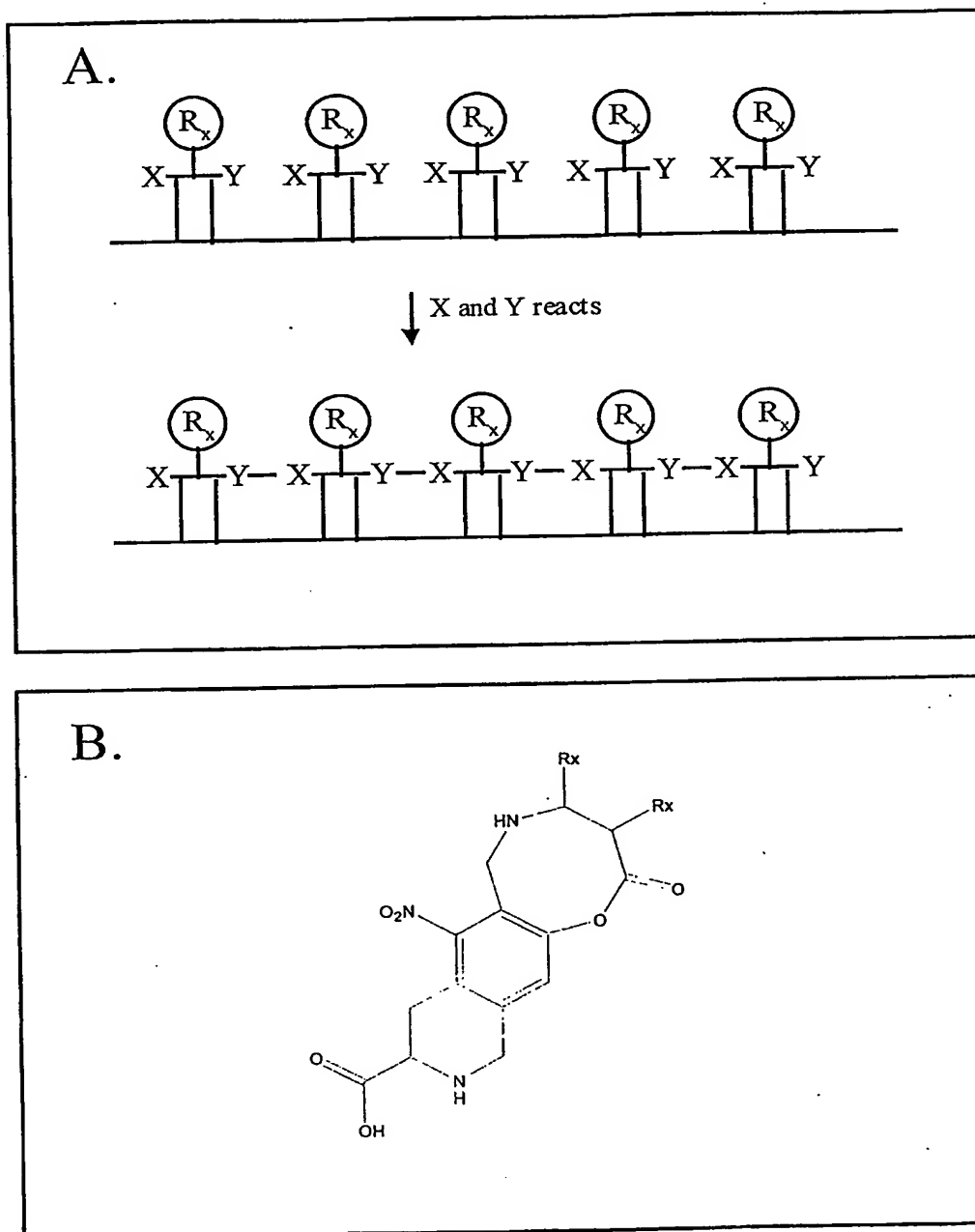
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Fig. 20. Zipping-polymerization and activation by ring opening.



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Fig. 21.
Directional polymer formation using fixed functional units.



55/68**Fig. 22. Templated polymers.**

- alpha-, beta-, gamma-, and omega-peptides
- mono-, di- and tri-substituted peptides
- L- and D-form peptides
- cyclohexane- and cyclopentane-backbone modified beta-peptides
- vinylogous polypeptides
- glycopolypeptides
- polyamides
- vinylogous sulfonamide peptide
- Polysulfonamide
- conjugated peptide (i.e., having prosthetic groups)
- Polyesters
- Polysaccharides
- Polycarbamates
- Polycarbonates
- Polyureas
- poly-peptidylphosphonates
- Azatides
- peptoids (oligo N-substituted glycines)
- Polyethers
- ethoxyformacetal oligomers
- poly-thioethers
- polyethylene glycols (PEG)
- Polyethylenes
- Polydisulfides
- polyarylene sulfides
- Polynucleotides
- PNAs
- LNAs
- Morpholinos
- oligo pyrrolinone
- polyoximes
- Polyimines
- Polyethyleneimine
- Polyacetates
- Polystyrenes
- Polyacetylene
- Polyvinyl
- Lipids
- Phospholipids
- Glycolipids
- polycycles (aliphatic)
- polycycles (aromatic)
- polyheterocycles
- Proteoglycan
- Polysiloxanes
- Polyisocyanides
- Polyisocyanates
- Polymethacrylates

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Fig. 23. Precursors - examples.

- N-carboxyanhydrides (NCA)
- N-thiocarboxyanhydrides (NTA)
- Amines
- Carboxylic acids
- Ketones
- Aldehydes
- Hydroxyls
- Thiols
- Esters
- Thioesters
- conjugated system of double bonds
- Alkyl halides
- Hydrazines
- N-hydroxysuccinimide esters
- Epoxides
- Haloacetyls
- UDP-activated saccharides
- Sulfides
- Cyanates
- Carbonylimidazole
- Thiazinanones
- Phosphines
- Hydroxylamines
- Sulfonates
- Activated nucleotides
- Vinylchloride
- Alkenes, quinones

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Fig. 24. Functional groups – examples.

- Hydroxyls
- Primary, secondary, tertiary amines
- Carboxylic acids
- Phosphates, phosphonates
- Sulfonates, sulfonamides
- Amides
- Carbamates
- Carbonates
- Ureas
- Alkanes, Alkenes, Alkynes
- Anhydrides
- Ketones
- Aldehydes
- Nitratates, nitrites
- Imines
- Phenyl and other aromatic groups
- Pyridines, pyrimidines, purines, indole, imidazole, and heterocyclic bases
- Heterocycles
- polycycles
- Flavins
- Halides
- Metals
- Chelates
- Mechanism based inhibitors
- Small molecule catalysts
- Dextrins, saccharides
- Fluorescein, Rhodamine and other fluorophores
- Polyketides, peptides, various polymers
- Enzymes and ribozymes and other biological catalysts
- Functional groups for post-polymerization/post activation coupling of functional groups
- Drugs, e.g., taxol moiety, acyclovir moiety, “natural products”
- Supramolecular structures, e.g. nanoclusters
- Lipids
- Oligonucleotides, oligonucleotide analogs (e.g., PNA, LNA, morpholinos)

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Fig. 25. Polymers and the functional entities required to make them.

A.

Polymer	Functional Entity (reactive groups)	Linking molecule	Catalyst/reagent	General Figure	Specific Figure
polycyclic compound	di-coumarin		light	Fig. 11	Fig. 11, ex. 1
polyester	alcohol, carboxylic acid		carbodiimide	Fig. 12, Fig. 21	
polyester	hydroxyl, thioester			Fig. 14	
polyurea	di-amine	carbonyldiimidazole		Fig. 15	Fig 15, ex. 3
polyacetate	halogen, carboxylic acid		base	Fig. 12, Fig. 21	
polyacetate	alcohol, carboxylic acid		EDC or other carbodiimide	Fig. 12, Fig. 21	
polycarbamate	alcohol, isocyanate			Fig. 12, Fig. 21	
polycarbonate	diol	carbonyldiimidazole		Fig. 15	
peptoid	secondary amine, α - haloacetyl			Fig. 12, Fig. 21	
	primary amine, α - haloacetyl		alkylating agent	Fig. 12, Fig. 21	
glycogen	UDP-glucose		glycogen synthetase	Fig. 12, Fig. 21	
polysaccharide	UDP-activated saccharides		polysaccharide synthetases	Fig. 12, Fig. 21	
polysaccharide	glucosyl sulphide/sulfoxide activation system (Kahne glucosylation)		Kahne conditions	Fig. 12, Fig. 21	
polyamide	amine, N- hydroxysuccinimide ester			Fig. 12, Fig. 21	
polyamide	amine, carboxylic acid		carbodiimide	Fig. 12, Fig. 21	

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Fig. 25, continued
Polymers and the functional entities required to make them.

B.

Polymer	Functional Entity (reactive groups)	Linking molecule	Catalyst/reagent	General Figure	Specific Figure
polyamide	di-amine	di-carboxylic acid	carbodiimide	Fig. 15	Fig. 15, ex. 2
polyamide	di-carboxylic acid	di-amine	carbodiimide	Fig. 15	
polyamide	amine, carboxylic acid	amine, carboxylic acid	carbodiimide	Fig. 16	
α -polypeptide	carboxyanhydride (5-membered ring)			Fig. 18	
β -polypeptide	carboxyanhydride (6 membered ring)			Fig. 18	Fig. 18, ex.1
γ -polypeptide	carboxyanhydride (7-membered ring)			Fig. 18	
α -polypeptide	2,2-diphenylthiazinanone (5-membered ring)			Fig. 18	
β -polypeptide	2,2-diphenylthiazinanone (6-membered ring)			Fig. 18	Fig. 18, ex.2
γ -polypeptide	2,2-diphenylthiazinanone (7-membered ring)			Fig. 18	
α -polypeptide	amine, thioester			Fig. 14	
β -polypeptide	amine, thioester			Fig. 14	Fig. 14, ex.1
γ -polypeptide	amine, thioester			Fig. 14	
ω -polypeptide	amine, thioester			Fig. 14	
polysulfonamide	amine, sulfonic acid		carbodiimide	Fig. 12, Fig. 21	
polyphosphonate	di-alcohol	activated phosphonate		Fig. 15	
polyphosphonate	di-alcohol	activated alkylphosphine	oxidating reagent, e.g. tert-butylhydroperoxide	Fig. 15	
polyphosphate	di-alcohol	diaminoalkoxy-phosphine	oxidating reagent, e.g. tertbutylhydroperoxide	Fig. 15	
polyphosphodiester	diol	diaminophosphine	oxidant (ButOOH)	Fig. 15	Fig. 15, ex. 5
polyphosphodiester	diaminophosphine	diol	oxidant (ButOOH)	Fig. 15	Fig. 15, ex. 6

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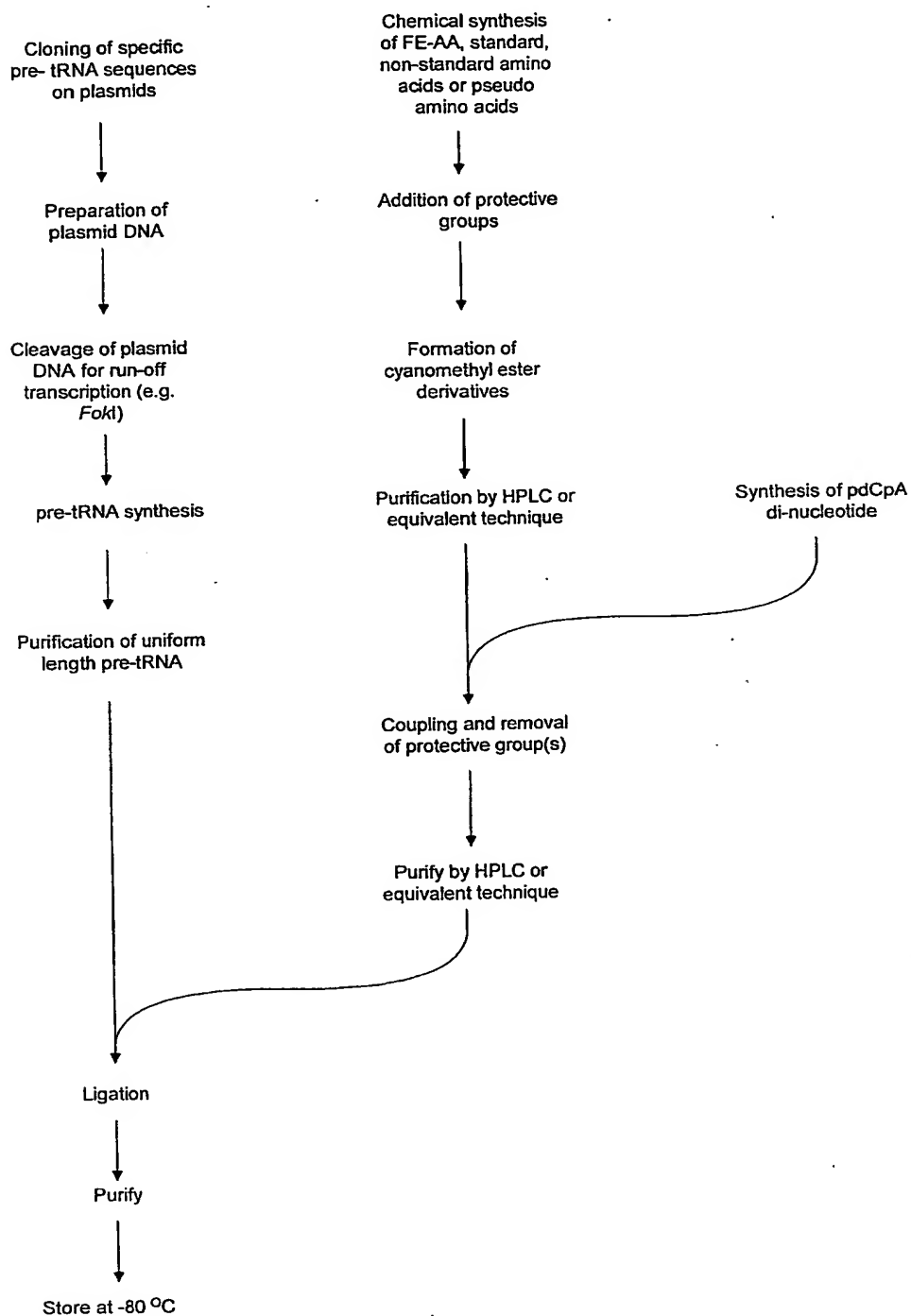
Fig. 25, continued

Polymers and the functional entities required to make them.

C.

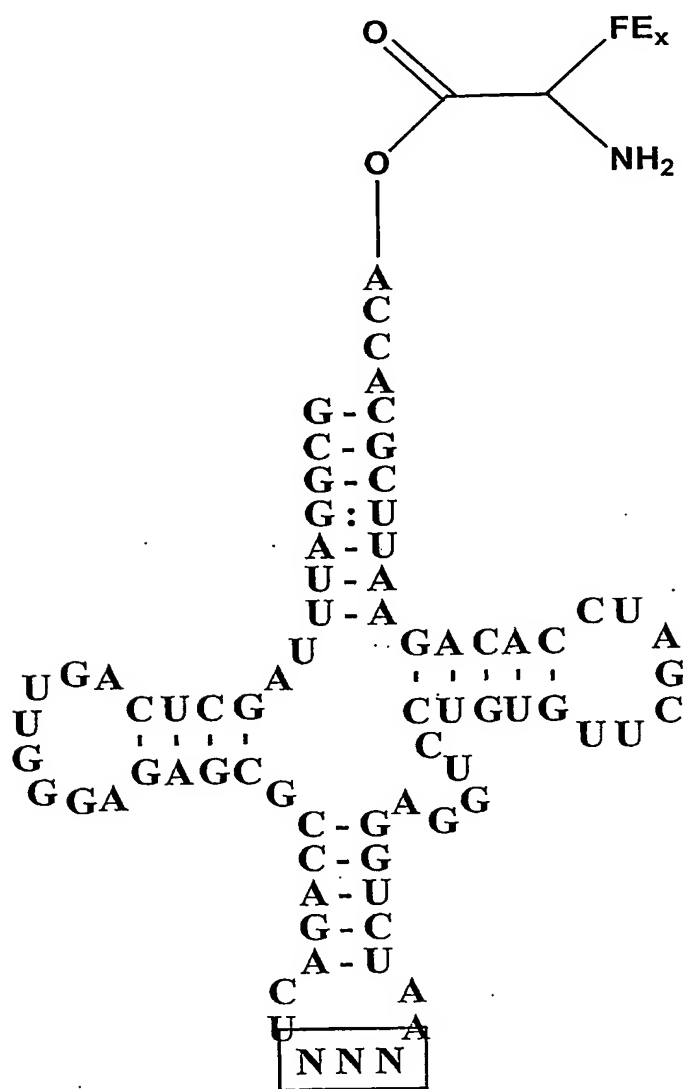
Polymer	Functional Entity (reactive groups)	Linking molecule	Catalyst/reagent	General Figure	Specific Figure
polyurethane	diamine	diisocyanate		Fig. 15	
				Fig. 18	Fig. 18, ex. 3
polyether	epoxide			Fig. 18	
polythioether	thioepoxide				
polydisulfide	thiol, thiol		oxidant	Fig. 11	
				Fig. 12, Fig. 21	
polyoxime	aldehyde, hydroxylamine			Fig. 12, Fig. 21	
polyimine	aldehyde, amine			Fig. 15	Fig. 15, ex. 1
polyimine	aldehyde, amine				
polynucleotides	nucleoside-5'-phosphoro-2-methylimidazolides			Fig. 12, Fig. 21	
polyamine	amine, alkyl sulfonate			Fig. 14	Fig. 14, ex.2
				Fig. 17	Fig. 17, ex. 1
alkane	alkene			Fig. 17	Fig. 17, ex.2
alkane	alkene				
polycycloalkane	di-diene	di-alkene (benzoquinone)		Fig. 15	Fig. 15, ex. 7
				Fig. 17	
polyvinyl	vinylchloride unit				
polystyrene	styrene-unit		radical initiator, AIBN	Fig. 17	
				Fig. 17	Fig. 17, ex. 1
polyethylene	ethylene unit				

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Fig. 26
Protocol for chemical charging of specific tRNAs



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Fig. 27A

An example of a general structure for a set of building blocks.

Variable sequence (i.e. anticodon)

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Fig. 27B

Examples of anticodon sequences and their corresponding functional entities

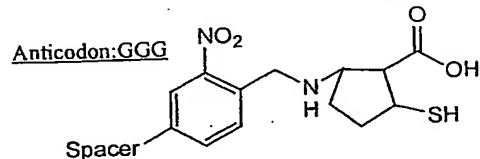
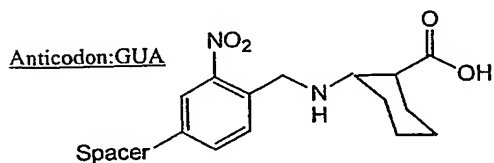
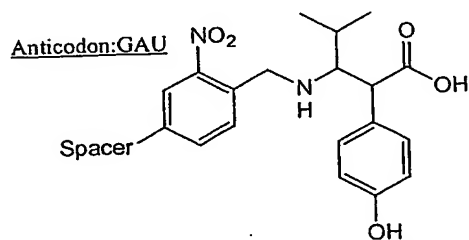
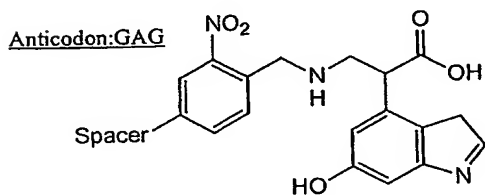
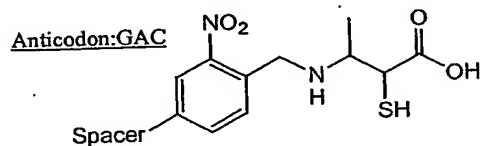
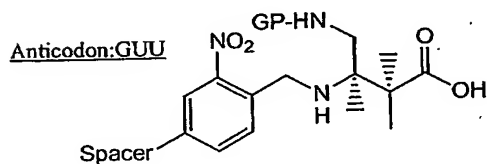
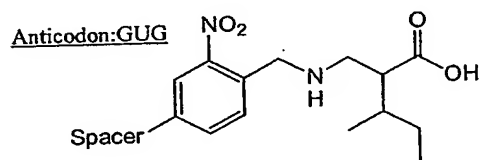
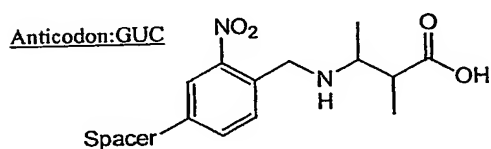
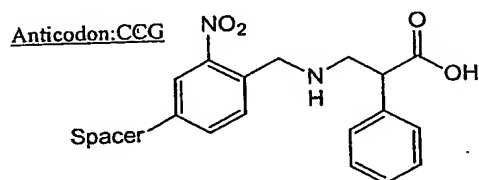
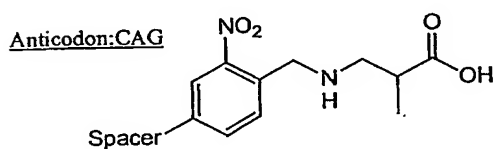
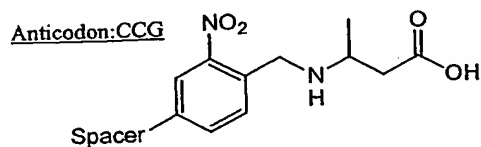
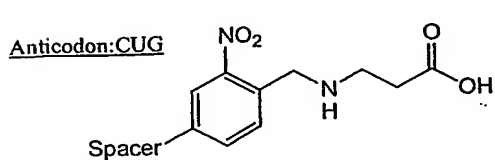
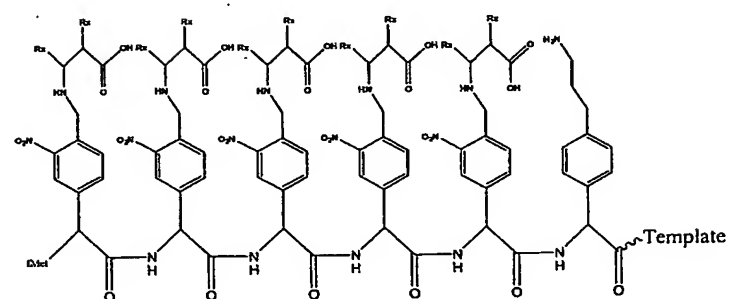


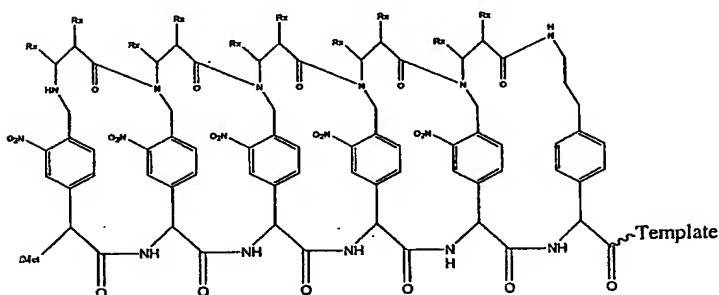
Fig. 28

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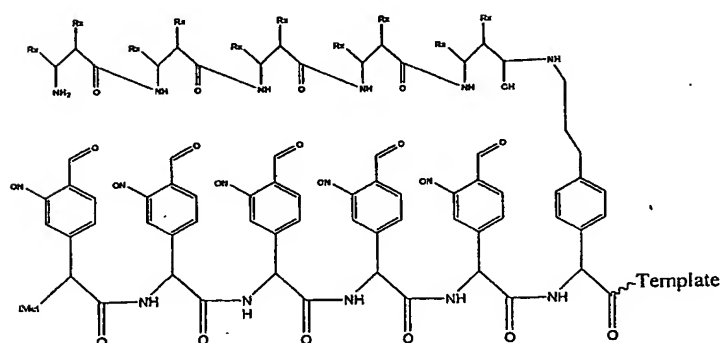
Bond formation and linker cleavage



EDC/NHS (pH 8.0)



Photocleavage of linkers (and protective groups)



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Fig. 29 Pairs of reactive groups X, Y and the resulting bond XY.

Nucleophilic substitution reaction









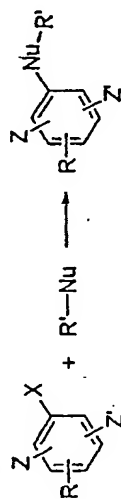
$R-X$	$+ R'-O^-$	\longrightarrow	$R-O-R'$	ETHERS	$R-C(=S)-O-R'$	$+ R''-NH_2$	\longrightarrow	$R-C(=S)-NH-R''$	THIOAMIDES
$R-X$	$+ R'-S^-$	\longrightarrow	$R-S-R'$	THIOETHERS	$R-C(=O)-O-R'$	$+ R''-NH_2$	\longrightarrow	$R-C(=O)-NH-R''$	AMIDES
$R-X$	$+ R'-NH_2$	\longrightarrow	$R-NH-R'$	sec-AMINES	$R-C(=S)-O-R'$	$+ R''-NH_2$	\longrightarrow	$R-C(=S)-NH-R''$	THIOAMIDES
$R-X$	$+ R'-N-R'$	\longrightarrow	$R-N-R'$	tert-AMINES	$R-X$	$+ R'-N(R)OH$	\longrightarrow	$R-N(R)OR'$	OXIMES
	$+ R'-O^-$	\longrightarrow	HO OR 	β -HYDROXY ETHERS	R^*-SO_2Cl	$+ R'-N-R'$	\longrightarrow	$R^*SO_2-N(R)R'$	SULFONAMIDES
	$+ R'-S^-$	\longrightarrow	HO SR' 	β -HYDROXY THIOETHERS	$R'-X$	$+ R'-\overset{\ominus}{C}(Z)_2$	\longrightarrow	$R'-C(Z)_2R'$	DI- AND TRI-FUNCTIONAL COMPOUNDS
	$+ R'-NH_2$	\longrightarrow	HO NHR' 	β -HYDROXY AMINES	$R'-C(=O)L$	$+ R'-\overset{\ominus}{C}(Z)_2$	\longrightarrow	$R'-C(Z)_2R'$	DI- AND TRI-FUNCTIONAL COMPOUNDS
	$+ R'-O^-$	\longrightarrow	RHN OR' 	β -AMINO ETHERS	$Z, Z' = COOR, CHO, COR, CONR^*_2, COO^*, NO_2, SOR, SO_2R, SO_2NR^*_2, CN, \text{ etc.}$				
$R-C(=O)-O-R'$	$+ R''-NH_2$	\longrightarrow	$R-C(=O)-NH-R''$	AMIDES					
$R-C(=O)-S-R'$	$+ R''-NH_2$	\longrightarrow	$R-C(=O)-NH-R''$	AMIDES					

Fig. 29, continued

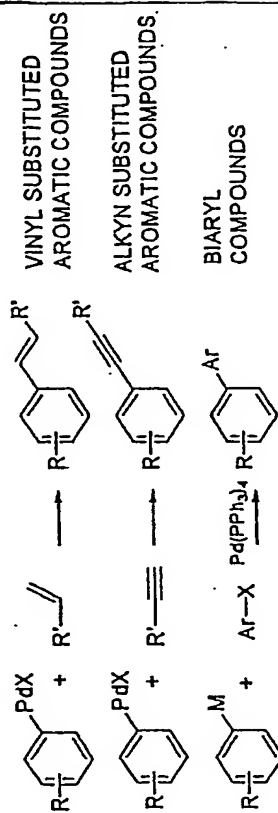
Aromatic nucleophilic substitution

SUBSTITUTED AROMATIC COMPOUNDS

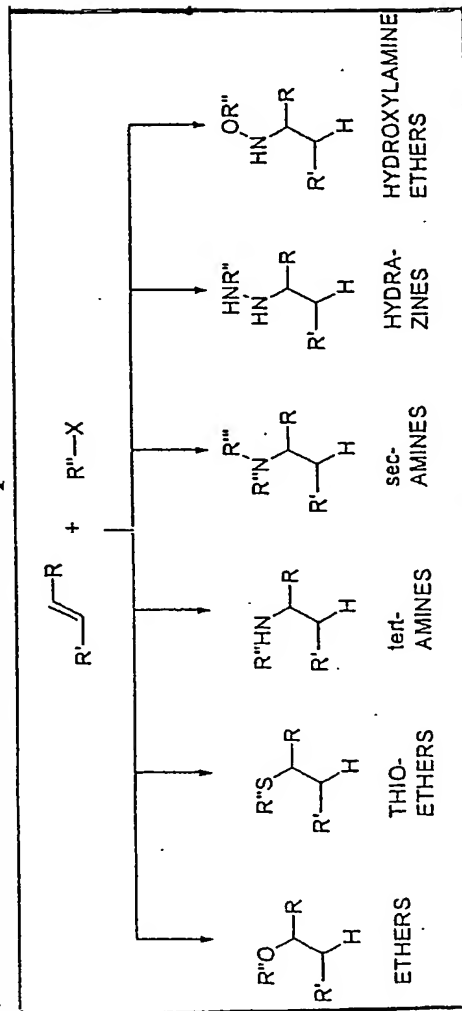


Nu = Oxygen-, Nitrogen-, Sulfur- and Carbon Nucleophiles
 X = F, Cl, Br, I, OSO₂CH₃, OSO₂CF₃, OSO₂TOL, ... etc.
 Z, Z' = COOR, CHO, COR, CONR'', COO⁻, CN, NO₂, SOR, SO₂R, SO₂NR'', ... ect.

Transition metal catalysed reactions

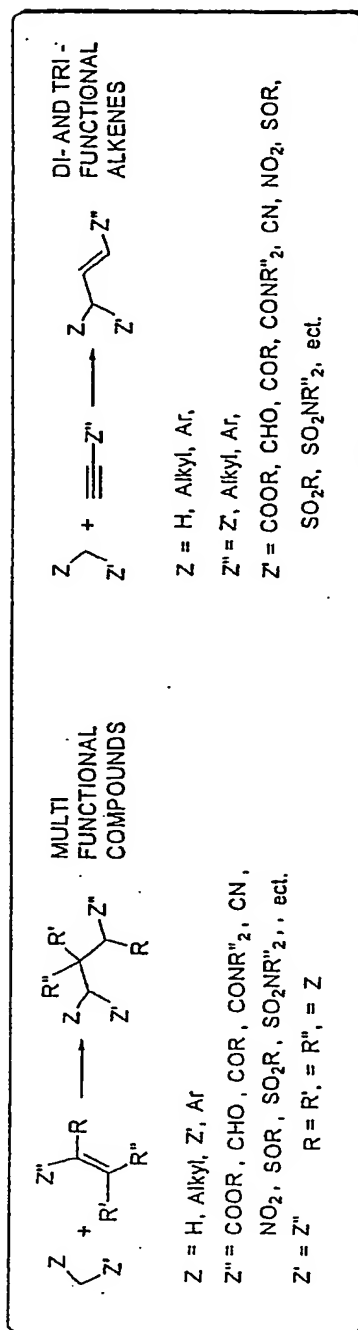


Addition to carbon-carbon multiple bonds

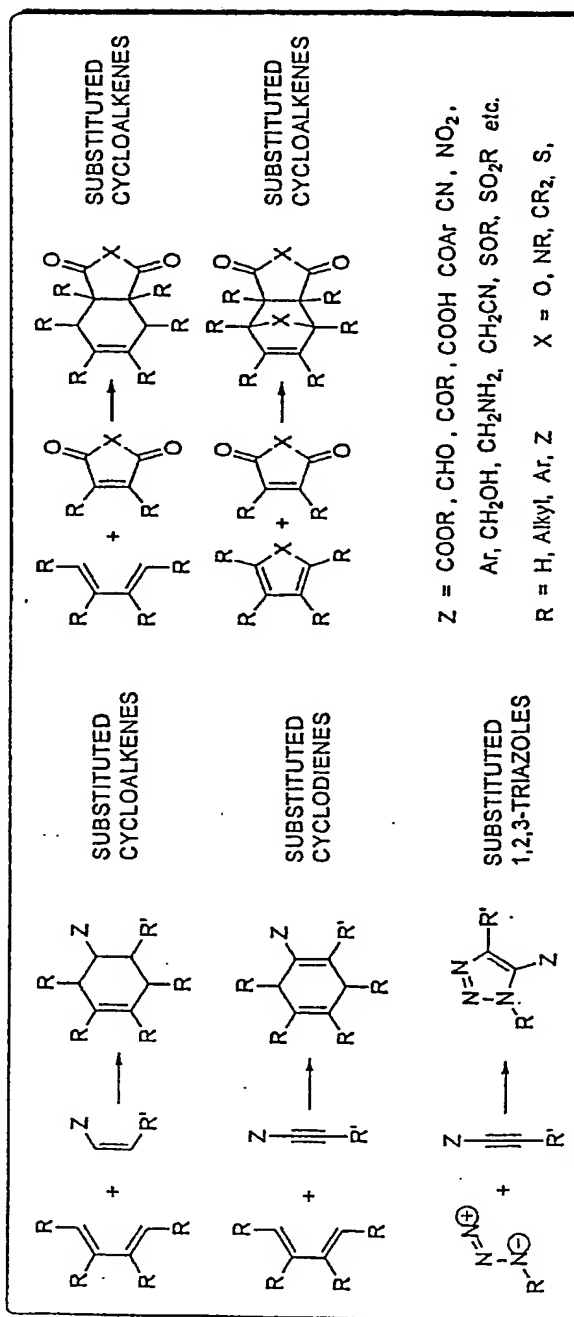


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Fig. 29, continued



Cycloaddition to multiple bounds



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